ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL INSTITUTES OF HEALTH

1967 - 1968

DIVISION OF BIOLOGICS STANDARDS

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United States. National Institutes of Health.

DIVISION OF BIOLOGICS STANDARDS

Report of program activities

ANNUAL REPORT

July 1, 1967 through June 30, 1968

TABLE OF CONTENTS

	Serial No.	Page
OFFICE OF THE DIRECTOR		
Introduction (Summary)		i
LABORATORY OF BACTERIAL PRODUCTS (LBP)		
Summary		1
Contract Narratives		
Columbia University (PH43-64-76)		9
Orentreich Research Corporation (PH43-64-885)		11
Michigan Department of Health (PH43-66-552)		13
University of California Medical School (PH43-68-632)		15
The Composition, Immunology, and Specificity of Tuberculin	100	16
Specificity and Hypersensitivity of Tuberculins	135	19
Immunology of the Delayed Contact Sensitivity Induced by Catechols Related to and Contained in Poison Ivy Extracts	102	22
Standardization of Allergenic Extracts	119	24
Standardization of Methods of Skin Testing Humans for Allergy	134	26
Mycotic and Parasitic Diagnostic Skin Test Reagents	103	28
The Pathogenesis of Lesions Induced by Antigens in Water-in-Oil Emulsions	120	30
Studies on Tuberculosis Vaccine	101	32
Investigation of Tetanus Toxoids for Prevention of Neonatal Tetanus	12	33
A Study of the Antigenic Substances of *Clostridium tetani*	13	35
Standardization of Reference Preparations for Tetanus Toxoids	14	37
Investigation of Toxicity of Oil Emulsified Antigens	15	39
Role of Transitional L-Forms of Bacteria in Human Diseases	16	41
Mycoplasma in Human Disease and Biologics	17	43
Studies on Cholera and Cholera Vaccine	22	45
Studies on Pertussis Vaccine	104	49
Studies on Typhoid Vaccine	116	51
Evaluation of Microbially Controlled Animals in Control Testing and Infection	136	54
Selective Breeding to Establish a "Standard" Mouse	138	57

RH
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	Serial No.	Page
LABORATORY OF BIOPHYSICS AND BIOCHEMISTRY (LBB)		
Summary		59
Search for Infectious Agents in Canine Cell Cultures by Fluorescence Microscopy; their Identification by Standard Virological Techniques and by Electron Microscopy.	157	61
Search for Adventitious Agents in Primary Simian Cell Cultures by Fluorescence Microscopy; their Identification by Standard Virological Techniques and by Electron Microscopy.	156	64
Detection and Characterization of Viruses	86	66
Development of Methods for the Chemical Analysis of Biological Products	29	71
LABORATORY OF BLOOD AND BLOOD PRODUCTS (LBBP)		
Summary		73
Contract Narratives		
Baylor University (PH43-65-1046)		79
University of Texas (PH43-68-97)		79
Mason Research Institute (PH43-67-676)		81
Wisconsin Alumni Research Foundation (PH43-67-684)		81
Preparation, Characterization, Stability and Potency of Blood Products	33	82
The Clotting and Fibrinolytic Systems	81	86
Proteins of Body Fluids	82	89
LABORATORY OF CONTROL ACTIVITIES (LCA)		
Summary		91
Contract Narratives		
Midwest Research Institute (PH43-67-679)		98
University of Oregon Medical School (PH43-67-675)		99
Southwest Research Institute (PH43-67-1104)		100
IIT Research Institute (PH43-67-1101)		101
Bacteroides vulgatus for Testing Fluid Thioglycollate Medium	143	102
Clostridium botulinum Toxin and Antitoxin	144	104
Coral Snake Venom and Antivenin	145	105
Standardization of Reference Diphtheria Antitoxin for Flocculation Test	146	107
Development of an Improved Opacity Standard	147	109
Development of Sterility Test Procedures for Testing Dried Smallpox Vaccine for Jet Gun Injection	148	111
Comparison of Titrations on the Chorioallantoic Membrane of Chick Embryos with the Rabbit Scarification Technique for the Potency Assay of Smallpox Vaccine	149	113
Replacement of the U. S. Standard Tetanus Antitoxin	150	115

	Serial No.	Page
Suppression of Tuberculin Skin Reactions	151	117
Statistical Evaluation of the Guinea Pig Potency Assay and Standardization of Old and PPD Tuberculins	152	118
Development of a Reference Typhus Vaccine and an Improved Potency Test	153	120
Bactericidal Activity of Wasp Extracts	154	122
RIF-free Yellow Fever Virus Seed	155	124

LABORATORY OF PATHOLOGY (LP)

Summary		127
Contract Narrative Bionetics Research Laboratories (PH43-67-1483)		131
Developmental Studies of Neurovirulence Testing on Poliovirus Vaccines in Monkeys	41	132
The Pathogenesis of Infectious Diseases	44	134
Histologic and Ultrastructural Development of the Central Nervous System and its Relationship to the Immunological Response	45	137
The Development of Toxic States	95	139
Breeding of Simian Primates	96	141
Developmental Studies for Control Testing of Attenuated Live Virus Vaccines	112	143
In vitro Study of Cells from the Central Nervous System	113	146
Studies of Tissue Transplants in Various Animals	114	148
Studies of Marmosets *Saguinus mystax* and their Suitability for Biologics Testing	133	150

LABORATORY OF VIRAL IMMUNOLOGY (LVI)

Summary		153
Contract Narrative Baltimore Biological Laboratory (PH43-66-1163)		161
Investigations Concerning Rubella Virus and Vaccine	49	163
Investigations Concerning Mumps Virus and Vaccine	48	170
Immunological Studies of Viruses Associated with Human Lymphoma and Leukemia	127	173
Investigations Concerning the Smallpox Group of Viruses and Smallpox Vaccines	47	175
Use of Tissue Culture in the Study and Diagnosis of Heritable Metabolic Disease	53	177
Investigations into the Etiologic Agent of Viral Hepatitis	137	179
Investigations Concerning Measles Virus and Vaccine	46	181
Studies on Simian Virus 40	52	182
Investigations Concerning Combined, Live Virus Vaccine	126	184
Studies on Infectious Viral Nucleic Acid	128	185
Demonstration of a New Congenital Anomaly in the Rabbit	129	186
Studies on Ferritin-Conjugated Antibodies	130	187

	Serial No.	Page
LABORATORY OF VIROLOGY AND RICKETTSIOLOGY (LVR)		
Summary		189
Contract Narrative		
Melpar, Inc. (PH43-67-1105)		197
Office of the Chief	57	198
Studies on Autoimmune Reactions Related to Viral and Adjuvant Sensitization	139	200
Studies on Agents of Chronic and Degenerative Diseases	140	202
Studies on Arthropod-Borne Viruses (Arboviruses)	75	204
Studies on Hemorrhagic Fever Viruses	118	206
Studies on Yellow Fever Vaccine	121	209
Studies on Respiratory Viruses. I. Influenza Virus Vaccines	122	211
Studies on Adenoviruses	123	213
Studies on Endogenous Viruses Contaminating Avian Eggs. I. Duck Hepatitis Virus	141	215
Avian Leukosis Viruses in Relation to Human Vaccines	117	217
Egg Yolk Antibody as a Tool to Study Avian Microbial Infections Potentially Contaminating Human Vaccines	125	220
Studies on Smallpox Vaccines and Vaccinia Virus	124	222
Antigenic Studies of Rickettsiae	59	224
Studies of the Nucleic Acids and Genetic Relatedness of Rickettsiae	82	226
Mechanisms of Intracellular Infection	65	228
Standardization Techniques Applicable to Large-Scale Production of Cell Cultures	67	230
Development of New Cell Culture Systems as Possible Substrates for Vaccine Production and/or Testing	79	232
Studies on the Biology of Cultured Cells	83	234
Identification of Specific *in vitro* Cell Differential Antigens	142	236
Studies Concerned with Influenza Viruses	60	238
Studies Concerned with Adenoviruses	98	243
Studies on Antiviral and Antitumor Agents	74	246
ADJUNCTS TO THE OFFICE OF THE DIRECTOR		
Experimental Virology	01	251
Biometrics Section		
Summary		253
Development of Statistical Methodology Applicable to Biologics Control	111	259
Investigational New Drug Office (IND)		263
Contract Operations Office		
Summary		265
Contract Narratives		
University of Michigan (PH43-67-1154)		266
IIT Research Institute (PH43-67-1106)		267

	Serial No.	Page
Melpar, Incorporated (PH43-67-1100)		269
Yale University (PH43-67-1463)		271
Woodard Research Corporation (PH43-67-1397)		273
Bio-Research Consultants (PH43-67-677)		274
University of Maryland (PH43-68-646)		275
University of Pittsburgh (PH43-67-701)		276
University of Minnesota (PH43-67-705)		276
Bionetics Research Laboratories (PH43-67-1483)		277
Studies of Virus-Cell Interactions	132	278
Interferon Production by Oncogenic Viruses	131	280
Information Office		283
Scientific Communications Office		
Summary		285
Contract Narrative		
Biological Abstracts, Inc. (PH43-67-74)		286

INDEXES

Contracts	291
Investigators	293
Publications	301
Subject	309

o OD
Introduction
ntracts
LBP
LBB
ators
LBBP
LCA
Publ
LP
LVI
ject Inc
LVR

D I V I S I O N O F B I O L O G I C S S T A N D A R D S

SUMMARY OF THE DIRECTOR

INTRODUCTION

The mission of the Division is the exercise of controls on the safety, purity, and potency of biological products through a system of licensing of manufacturing establishments and of the products, as well as the conduct of research related to these objectives in order that decisions made and actions taken can be based on sound information.

Basically, it is the responsibility of the Division to make recommendations for standards for all biological products such as vaccines, serums, human blood and its derivatives, and like products. These include written standards, eventually promulgated as regulations, some of which apply to individual products and some to products in general; and physical standards, i.e., biological preparations whose properties and qualities have been extensively characterized. The latter are issued to manufacturers and other research laboratories concerned with biologics standardization for evaluation with their products, particularly with respect to potency, or strength.

The acquisition of the information needed to develop written standards and the laboratory investigations leading to the development and selection of suitable physical standards are extensive in character and often time-consuming. This is particularly true of standards for the tissue-culture-prepared virus vaccines such as poliovirus, measles, and mumps which have been developed in recent years.

When a new vaccine against a particular disease appears achievable, it devolves upon the Division to develop standards that will be ready at approximately the same time that the product is brought to the final stages of manufacture. This requires considerable research involving most of the component laboratories of the Division, as well as the testing of samples of both experimental and final products. It is necessary during this period to maintain close contact with the manufacturer and with scientists working in the field so that problems related to standards can be ironed out during the developmental phases. In addition, any clinical investigations conducted by the manufacturers or by investigators studying the products under a variety of conditions come under review of the Division.

When a manufacturer has decided to market a product which he has developed, he submits a license application to the Division together with samples of the product and results of all tests. Division scientists then review the

the application and examine the product in the laboratory to ascertain the accuracy of the manufacturer's test results. If the tests fulfill the requirements and the application is in order, the product license is recommended for approval.

In the case of a manufacturer already licensed for other biological products, production facilities may be inspected as deemed necessary according to the nature of the pending product. In the case of a new manufacturer, production facilities are inspected and the personnel assessed prior to licensing the manufacturing establishment, as well as the product he proposes to market. The establishment is inspected annually thereafter to verify that proper procedures are being followed in manufacturing and testing procedures. Samples and the results of tests performed on each batch or lot of all biological products proposed for marketing must be submitted to the Division by the manufacturer prior to distribution. The Division checks the test results and runs similar tests to ensure the quality of the products. Only then is a release for marketing issued to the manufacturer.

Once a product has come into use, its safety and potency must be continuously monitored. The knowledge and skill required to do this also provides a sound basis for consideration of problems associated with newer products as they are developed.

Brief reviews of the control activities, and of licensings, inspections, investigations, and legal actions are included in this summary. It is noteworthy, however, that except for the staff involved in licensing and investigations, the scientific personnel of the Division's laboratories participate in inspections and control tests as well as in the research which comprises the main substance of this report.

Continued research on both old and new products is necessary not only for obtaining information leading to development of better standards, but also for the development of expertise and scientific competence on the part of the staff. In many respects this is a more compelling inducement towards compliance than mere recourse to regulation-based actions.

Because of the Division's concern with products, as such, and because of the involvement of research staff in the regulatory function, a great deal of attention is directed to "applied" research problems. However, there is probably no organization within the NIH where there are more examples of the failure of the words "applied" and "basic" truly to define research endeavor. The problems of the Division involve advanced concepts of modern microbiology, molecular biology, protein chemistry, hematology, allergy and immunology. The scientific staff continually studies new methods and new approaches to the problems associated with biological products, because it must remain abreast of current research and be aware of the significance of new knowledge in relation to these products. Of major concern are the viral vaccines, some of which, like vaccinia and rabies, are among the oldest licensed products, while newer ones are continually being developed. The Division must attempt to evaluate the significance of the demonstrated potentiality of some viruses to produce tumors in animals under special circumstances; it must also be concerned

with the problems of covert viruses contaminating cell cultures and other systems used for the propagation of viruses. This requires that old products be reexamined in terms of scientific advances from time to time.

The Division arranged for a "Conference on Cell Cultures for Virus Vaccine Production" at the Clinical Center, National Institutes of Health, in November, 1967. Dr. Donald Merchant of the University of Michigan working through a contract between DBS and the University was the organizer and chairman. The papers presented indicated the great need for controlled, well-studied, uncontaminated cell cultures capable of supporting the growth of a wide spectrum of viruses. Such cultures must be free from adventitious agents and have a normal cell structure. The proceedings of this conference will be published, as a monograph, by the Journal of the National Cancer Institute.

The development of new respiratory viral vaccines, and the continual assessment of old products such as inactivated influenza requires the Division to develop knowledge concerning antibodies involved in protection of the respiratory tract. The characterization of antibodies in serum in comparison with those in respiratory tract secretions, and the development of knowledge on the sites of production and on the transport of these substances across cell membranes are important facets of immunity against infection with these viruses. Analogous problems exist in regard to the factors possibly related to hypersensitivity which have apparently contributed to severe illnesses resulting from natural infection following artificial immunization with some newly produced experimental inactivated respiratory vaccines, produced from such agents as respiratory syncytial virus and *Mycoplasma*. These are also related to cellular aspects of immunity, hypersensitivity, and allergy, all of which are problems attracting the attention of DBS scientists.

The study of blood proteins also extends beyond their relation to infectious diseases because of the Division's concern with blood and blood products. Work continues on the purification of various plasma components essential to the clotting and fibrinolytic systems.

Bacterial vaccines also continue to be a Division concern. Proponents of pneumococcal vaccines are presenting a case that antibiotic therapy cannot work fast enough to save debilitated patients from succumbing to pneumococcal pneumonia. New developments on purification of streptococcal M protein point towards the possibility of the development of vaccines for the prevention of rheumatic fever and rheumatic heart disease, also proposed on the basis of inadequacy of antibiotic therapy alone. Vigorous research on cholera indicates the likelihood that a toxin or toxins produced by the vibrio will be obtained in quantity and serve as the basis for the production of new immunogenic agents. DBS staff members are involved in the cholera investigations and must be prepared for the other developments mentioned above. At the same time, the Division is still confronted with problems of improving pertussis and tuberculosis vaccines, among others.

Another area of current concern and interest to both the Division's and the manufacturers' investigators is that of *Mycoplasma*. Some of these organisms have been incriminated as causes of disease in man. The research

currently being conducted on *Mycoplasmas* is of considerable importance since it has been demonstrated that these organisms grow readily in the cell cultures used for producing many virus vaccines.

Two years ago Division scientists succeeded in attenuating the rubella virus. Using this weakened virus they developed an experimental vaccine against rubella or German measles. Infections of this disease during early pregnancy frequently result in spontaneous abortions, still-births or birth of infants with grave defects such as blindness, deafness, heart disease, and brain damage.

Vaccines prepared from an attenuated virus have now been administered to thousands of persons. None of these individuals experienced vaccine-associated reactions; over 90 percent developed protective immunity. Continued observation of the first groups of children vaccinated two years ago indicates that the immunity conferred by the attenuated virus will be of long duration. Thus far, the vaccine appears safe as well as effective.

New techniques of administration of vaccines and other biological products also add to the practical problems of the staff, since these require careful appraisal. In addition, proposals for the use of mineral oil and other adjuvants in immunization and in hyposensitization have prompted the Division to mount an extensive series of contractual projects to help resolve the issue of the safety of these materials.

The scientific pursuits of the DBS staff represent responses to several factors. To some extent, the staff must initiate investigations because of proposals for new products being developed by other organizations. To some extent, the control functions lead the staff to recognize gaps in existing knowledge of various biological products and their effects. To a great extent DBS scientists, like their colleagues in other organizations, are motivated by adding to the body of knowledge in their fields.

SCOPE OF CONTROL TESTING PROGRAM

Unlike, last year's report, the functions of the Laboratory of Control Activities are described in the same format as the research laboratories--a laboratory chief's summary precedes individual project reports. Two tables have been included here to summarize the Division-wide effort.

Essentially the control testing program operates as follows: Manufacturers of a licensed product submit to the Division samples from each lot of the product together with protocols containing the results from all tests performed on that lot. Some 297 different biological products are currently licensed. Samples and protocols are submitted on approximately 178 of these.

Each protocol is reviewed by the Division to determine if the manufacturer's test results conform with established standards. In addition, some or all of the tests required on a particular product may be performed by the Division. If the manufacturer's test results and the results of tests performed by the

Division conform with standards, a letter of release for that lot is sent to the manufacturer. Only then may the manufacturer distribute this particular lot for sale.

During the 12-month period covered by this report, 3,899 lots of biologic products were released, 38 lots were rejected, and 59 lots were withdrawn by the manufacturer. Table 1 reflects the number of lots released by categories of products.

A protocol may consist of considerable information requiring up to 200 pages, for example, on a lot of Poliovirus Vaccine, Live, Oral, or data on only two pages for a lot of blood grouping serum. All protocols are recorded and reviewed by members of the Laboratory of Control Activities. The protocols are then forwarded to other Laboratories within the Division for review; the nature of the product determines the Laboratory(s) consulted. A protocol for Measles Virus Vaccine, Live, Attenuated, may be reviewed by personnel in six of the Division's Laboratories.

Some of the Division personnel are involved full-time in the control program, while many other individuals are involved on a much smaller scale. As the control testing on products becomes more specialized, a larger number of the research scientists are becoming involved in control activities.

Table 2 shows the total number of control tests performed by Division personnel last year. This gives no idea of the total effort involved, however, since tests requiring 15 minutes or less are given the same weight as those taking a month or more to complete. Continual attention is given to test development, and progress on these endeavors is to be found in various project reports of the different Laboratories. It is in this area, especially, that staff of the Laboratory of Control Activities are working in collaboration with other Division scientists.

The control activities of the Division also require the maintenance of a large number of different standard and reference materials. These are prepared in the Division or obtained by purchase. They are all tested in the Laboratories of DBS and distributed as needed. During fiscal 1968, 9,429 such preparations were sent out.

This illustrates the close relationship which the Division has built up between itself and the pharmaceutical manufacturers in order to maintain the high standards for biological products needed to protect the health of the community. In this respect, the Division again does more than await developments made elsewhere. Industry looks to the Division for the guidelines necessary for improving the quality, and maintaining the safety of biological products.

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TABLE 1

Project Group	Lots Released	Lots Rejected	Lots Withdrawn	Totals
Antitoxins	16	0	0	16
Therapeutic Serums	5	0	0	5
Blood and Blood Products	1,412	2	18	1,432
Bacterial Vaccines	127	1	2	130
Toxoids and Toxins	89	0	0	89
Multiple Antigen Preparations	99	4	4	107
Viral and Rickettsial Vaccines	780	7	13	800
Diagnostic Substances for Dermal Tests	149	0	0	149
Diagnostic Substances for Laboratory Tests	1,093	24	22	1,139
Miscellaneous	129	0	0	129
Totals	3,899	38	59	3,996

TABLE 2

TOTAL CONTROL TESTS PERFORMED IN REPORTING PERIOD

Type of Test		Number of Tests
Potency	In Vivo	2,080
	In Vitro	9,180
Safety	General	820
	Specific	7,418
Purity		10,291
Sterility		6,097
Total Control Tests		35,886

LICENSING, INSPECTIONS AND INVESTIGATIONS

The Licensing and Investigations Section of the Office of the Director has as its principal responsibility the correlation of licensing and enforcement activities arising from the Division's responsibilities under the Public Health Service Act 42 U.S.C. 262. The following statistics summarize the activities of the units for Licensing, Inspections, and Investigations.

LICENSING

Licensing actions are based on the recommendations of license review groups appointed by the Director from the scientific staff concerned with the particular type of product or establishment.

On April 1, 1968, 220 establishment licenses were in effect, together with 101 subsidiary establishments and 251 donor centers. This represents a change of 5 establishment licenses since April 1, 1967, the date taken for the last report. This difference is due to the issuance of 23 new licenses and the revocation without prejudice of 18 licenses. No licenses were revoked for cause during this period.

During the 12 months covered in this report, 144 product licenses were issued, 143 were revoked without prejudice, and none were revoked for cause. As of April 1, 1968, 1,322 product licenses were in effect for 297 different licensed products.

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There were 23 applications and 8 amendments for establishment licenses, and 99 applications and 49 amendments of product licenses pending at the time of this report.

During the year, the official name of blood issued for transfusion purposes was changed from Citrated Whole blood (Human) or Heparinized Whole Blood (Human) to Whole Blood (Human) to be preceded by appropriate terms describing the anticoagulant used. All product licenses bearing the previous terminology were revoked without prejudice and reissued using the new "proper name."

Notable among the product licenses issued during the year are 4 which are new products:

MUMPS VIRUS VACCINE, LIVE
MEASLES-SMALLPOX VACCINE, LIVE
ANTIVENIN (*Micrurus fulvius*)
ANTI-Rh_0 (D) GLOBULIN

INSPECTIONS

Forty-six members of the scientific staff, selected by the Laboratory Chiefs and the Office of the Director for their special knowledge, are authorized by the Secretary, Department of Health, Education, and Welfare to serve as inspectors. These individuals perform inspections as planned and coordinated by the Licensing and Investigations Section of the Office of the Director. Twelve additional professional employees assist in the inspections.

Between July 1, 1967 and May 1, 1968, 72 of 83 scheduled inspection trips were completed. During these trips 207 licensed establishments, 91 locations, and 133 blood donor centers were inspected for a total of 431 inspections. In addition 17 unscheduled trips were taken to inspect 13 establishments for licensing and 21 reinspections.

To accomplish the scheduled inspections, 476 man-days were required and 36 man-days for the unscheduled inspections. The remaining 11 scheduled trips will require 76 man-days for 57 inspections.

SUMMARY OF INSPECTIONS FOR FISCAL YEAR 1968

Scheduled Trips Completed	72	Scheduled Trips not Completed	11
Licensed Establishments Inspected	207	Licensed Establishments to be Inspected	35
Locations Inspected	91	Locations to be Inspected	7
Donor Centers Inspected	133	Donor Centers to be Inspected	15
Pre-Licensing Inspections	13		
Man-Days	476	Man-Days Anticipated	76

In support of its control activities the Division carries on an inspector-training program. New staff members from the various laboratories participate each year in a series of seminars. These sessions deal with the responsibilities of the Division, as defined in the Public Health Service Act, with licensing requirements for establishments, and with criteria to be used on inspections.

INVESTIGATIONS

Investigations are conducted by inspectors from the Office of the Director. In contrast to inspections, which call for expenditure of predictable periods of time, investigations must necessarily be carried out for as long and indefinite a period as is required to obtain all the needed information on a reported or discovered possible infraction of the law. Investigations must therefore be carried out by people who have this duty as their main function. In some situations, however, it is also necessary to call upon Laboratory resources for assistance.

From April 1, 1967 to April 1, 1968 investigations and legal actions involved a total of 51 man-days on travel status. Thirty-eight man-days were spent visiting both licensed and unlicensed establishments for enforcement purposes. The remaining 13 man-days were devoted to interviews and consultations with distributors, State health officials, and others.

During the report period covered here, the following actions resulted from Division investigations:

Legal Actions: (1) An appeal is still pending for one defendant in a case involving a blood bank. (2) Recommendation for revocation of a blood bank license is awaiting the Secretary's approval.

Voluntary Compliances: Four voluntary agreements of compliance were received from corporations and individuals who were operating in violation of the Public Health Service Act.

Blood Donor Study - Atlanta, Georgia: It has long been suspected that some blood donors will donate more often than the regulations now allow, and although there is some evidence of this, it remains sketchy and incomplete. An opportunity to examine this situation occurred in Atlanta when at the suggestion of the Division a system of voluntary donor identification had been established by four blood banks to prevent too frequent donation at different blood banks.

The microfilmed records of the four blood banks pertain to approximately 43,000 documents from which it is expected that nearly 37,000 keypunched cards representing the donations of Whole Blood (Human) and Plasma, and donor rejections in Atlanta, Georgia between January 1 and September 17, 1967 will be obtained.

The information from the Atlanta donor pool is small enough to handle easily, and offers the unique opportunity to determine the frequency with which donors give blood at different blood banks in a large metropolitan area. Information from this study might be helpful as a guide in the formation of new regulations, and possibly more effective administration of the present ones.

OFFICE OF THE DIRECTOR

SUMMARY

Program direction and administration for the Division of Biologics Standards is based in the Office of the Director, with centralization of administrative procedures relating to personnel, supply, and accounting. Major objectives are determined and program responsibilities are assigned to the operating units within the Division. For each major area, staffing patterns are developed and are continually re-evaluated in relation to program changes. The required support is determined for each area in relation to the Division's total budget. A major activity of the Director's office is the maintenance of the proper balance between control activities and research programs.

The Office of the Director includes an Information Office, a Biometrics Section, an Investigational New Drug Office, a Scientific Communications Office, and an Experimental Virology Section.

The Information Office is the means for communicating to the public significant aspects of the Division's research and control activities, responding to requests for information from the general public and for reports to higher echelons within the Public Health Service and the Department, and to the Congress.

The Biometrics Section is set up within the Director's Office because its functions cut across the entire Division. The tasks of this section represent the performance of work, using already established statistical techniques, for solving problems related to vaccine-testing originated in various Laboratories. In addition, the Section is involved in studies directed toward perfecting statistical methods used in bioassays and to serving the

research needs of the Division. This involves the problems of acceptance sampling and contingency table analysis, relating respectively to the laboratory examination of vaccines for safety and to the evaluating of field trials of candidate vaccines.

The "IND" Office receives and examines "Notices of Claimed Investigational Exemption for a New Drug," which are required by section 130.3 of the Food and Drug Regulations, has continued to function within the Office of the Director. During the period covered by this report, 61 "Notices" were received. This brings to 305 the total number of "Notices" received since the Food and Drug Regulations requiring submissions became effective. The Office has carried on an extensive correspondence to correct deficiencies in submissions and to point out possible hazards. The "Notices" add to the Division's alertness regarding the development of new products. The staff had opportunity to "tool up" in advance for the formulation of regulations and standards for such new products through this mechanism.

Data and information pertaining to licensed biological products and to new products in the offing are of immediate and direct importance to the regulatory and control programs. The Office of Scientific Communications performs bibliographic and reference services for the Division and maintains current awareness procedures designed to retrieve selected types of information for members of the staff. It is also working on an effective and more rapid means for processing information. Since the entire field of biomedical research requires new information retrieval techniques, such developments, for DBS, proceed along with systems that are being worked out for the whole body of knowledge. This requires close contact with information specialists working elsewhere within NIH and in other agencies.

ORGANIZATIONAL CHANGES

The formalization of certain organizational changes, directed toward better functioning of the Division, took place during the period covered by this report. Dr. John C. Wagner, who for several years has been acting in a dual capacity, was able to relinquish his additional assignment as Acting Chief of the Laboratory of Control Activities when Dr. Edward B. Seligmann was apointed Chief of that Laboratory. Dr. Seligmann will continue to act as Chief of the Reference Standards Section, LCA. For the purpose of centralizing responsibility for Protocol Review, and to provide staff support to the Assistant Director for Release Activities, a Section on Protocol Review and Product Release was created in the Office of the Director. Mr. Robert W. Kolb was designated Chief of that Section. Mr. Green replaced Mr. Kolb as Chief of the Control Test Section, in the Laboratory of Bacterial Products. Dr. Michael Barile, was designated Chief of a new Section on Mycoplasma and Dr. Sotiros D. Chaparas was designated Chief of a new Section on Mycobacterial and Fungal Antigens. In addition, Dr. M. Carolyn Hardegree was designated Chief of the Bacterial Toxins Section in the same laboratory. Dr. Ronald Clark was designated Chief of the Histopathology Section in the Laboratory of Pathology. Dr. Pinya Cohen transferred from the Laboratory of Control Activities to the Laboratory of Blood and Blood Products becoming Chief of the Section of Blood and Blood Derivatives. Since Dr. C. W. Hiatt's

resignation as Chief of the Laboratory of Biophysics and Biochemistry, Dr. Roderick Murray has been Acting Chief of that laboratory. Likewise, Dr. Sam T. Gibson has been Acting Chief of the Laboratory of Blood and Blood Products since the resignation of Dr. E. H. Mealey.

PERSONNEL CHANGES

Dr. John T. Tripp, Assistant to the Director, resigned in November, 1967 to accept a position as Director of Special Services at Blood Services in Scotsdale, Arizona.

Dr. Leon Jacobs accepted a post in the Office of the Assistant Secretary for Health and Scientific Affairs, DHEW.

Dr. Amos L. Palmer, a veterinary officer in the Laboratory of Pathology, resigned to return to private practice.

Dr. Hector Montes de Oca, a visiting scientist from Argentina, accepted a position in the Department of Anatomy at the University of Saskatchewan when his appointment expired in December, 1967.

There have been a number of new appointments to the staff. Dr. Jose G. Canchola and Dr. Paul Albrecht, both research biologists, have been assigned to the Laboratory of Virology and Rickettsiology. Dr. Henry C. Orr, a Staff Fellow, was appointed Acting Chief of the Tissue Culture Section in the same laboratory. Dr. Charles R. Manclark and Dr. Douglas Lorenz, both research microbiologists, have been assigned to the Laboratory of Bacterial Products and the Laboratory of Pathology, respectively. Dr. Carl E. Miller, a research veterinarian, assigned to the contracts program to provide guidance for testing projects involving animals, also gives support to the basic research programs of the Division.

HONORS TO STAFF MEMBERS

Dr. Harry M. Meyer, Chief of the Laboratory of Viral Immunology, and Dr. Paul D. Parkman of the same laboratory, received the E. Mead Johnson Award for Pediatric Research for 1967. In addition, Dr. Parkman was selected by the Jaycees as one of America's Ten Outstanding Young Men for 1967. Dr. Bernice E. Eddy, Chief of the Experimental Virology Section, received the Department's Superior Service Award at a Public Health Service ceremony. Dr. Margaret Pittman, Chief of the Laboratory of Bacterial Products, was elected to her second 4 year term on the Board of Governors of the American Academy of Microbiologists.

Many other members of the Division have been recognized for their special competences, as indicated by invitations to lecture, to serve on scientific committees, study groups, etc., or as leaders of symposia.

CONTRACTS PROGRAM

During the past three years a contracts program, established to serve as an extension of, and supplement to, the Division's research and control activities, has undergone rapid, but ordered growth.

The Division presently supports twenty-two active contracts, the majority of which are directly related to questions involving oncogenesis of viral vaccines and carcinogenic or toxic properties of vaccine components, adjuvants or other Biologics.

It is anticipated that the present level of contract activities will remain relatively constant as project termination balance new project needs.

Details of all individual contract-supported projects will be found in the "Contract Narratives" following each Laboratory Chiefs' summary, in those instances where Project Officers are assigned from the individual laboratories to manage the projects. Other projects, initiated and managed by the Contracts Staff of the Office of the Director, are included under "Adjuncts to the Office of the Director." All contracts are listed in a special index to this report.

Although the contracts staff personnel participate extensively in the supervision of contract operations, providing materials for tests and examining results of day-to-day activities, the scientific staff of the Division also contributes greatly to the development and function of the contracts program. This contribution has been of particular value in the initiation, supervision and evaluation of the contract efforts. Often this has involved considerable expenditure of time, made at a sacrifice to their own programs. Members of the contract staff are also engaged in direct research.

RESEARCH ACTIVITIES

The following summary of the program of the laboratories reflects the broad interests of Division scientists. For convenience, the work to be described will be identified only with the laboratory of the principal investigator. There is, however, a considerable amount of inter-laboratory collaboration. Consult the index to investigators appended to this report.

SECTION ON EXPERIMENTAL VIROLOGY (OD)

This section devotes its attention to viral carcinogenesis, with particular emphasis on biological factors mediating the extent to which neoplastic changes can be enhanced or suppressed.

The amount of SV_{40} virus injected into newborn hamsters was found to be a determining factor in the number of appearance of tumors. Fewer tumors developed in animals given large inocula than in those which received small

doses. These results were obtained when daily inoculations were made over the first 6 to 10 days after birth. They suggest that within this short period of time the larger doses stimulate antibody-production which prevents tumor development.

Apparent suppression of Simian virus 40 (SV_{40})-induced tumors in newborn hamsters by subsequent repeated injections of one strain of adenovirus 12 is described. Another adenovirus 12 strain failed to show this suppressive effect under similar conditions. Exploratory experiments of this type have been continued with a number of other adenoviruses. One strain, a type 4, which had never been propagated in monkey kidney cells where it might have been in contact with SV_{40}, appears to have a suppressive effect in the same system. Strains of other types of adenovirus gave equivocal results.

Some evidence was obtained that normal hamsters have a greater resistance to transplanted neoplastic cells than do hamsters already bearing tumors induced by the homologous virus. Transplanted cells induced tumors earlier in the tumored animals than in the normals.

LABORATORY OF BACTERIAL PRODUCTS (LBP)

Tetanus studies: The cooperative study on the prevention of neonatal tetanus in New Guinea by injection of various types of tetanus toxoid preparations is nearing completion with the final collections of sera to determine duration of antitoxin levels and booster response to adsorbed and plain toxoids. The results indicate that women of childbearing age given a primary immunization of two injections of adsorbed toxoid followed by a single booster of adsorbed toxoid at the time of pregnancy would have sufficient antitoxin levels to protect their newborn against tetanus. In the next phase of the study to be performed at DBS, effort will be directed towards relating the findings to a quantitative potency assay for tetanus toxoids and the assessment of the need to specify a unitage requirement in place of the present minimum potency requirement.

Water-in-oil adjuvants: Earlier work showed that although abscess formation of antigen in water-in-oil adjuvant and Arlacel A paralleled the free fatty acids content, the free fatty acids, per se, were not responsible for the abscess formation. In recent work it has been shown that the components of oil adjuvants, especially Arlacel A, are capable of inducing cytopathic effect in tissue cell cultures. The degree of the cytopathic effect has correlated well with toxicity measured by the intraperitoneal mouse test. The results were reproducible in six cell line cultures. These early findings indicate that cell cultures will be a valuable tool for measuring the toxicity of oil adjuvant components. Results are being compared with the findings of the various DBS contracts which are directed towards the chemical analyses and the biological and immunological effects of these components.

In the study on the separation and characterization of antigens in filtrates of *Clostridium tetani*, an esterase activity detected by the use of Arlacel A, olive oil and peanut oil was found to be enhanced by the addition of 7S gamma globulin. The activity is not related to the level of antitoxin added. The

mechanism of the reaction and its significance especially in relation to untoward reactivity of oil-in-water adjuvants remains to be determined.

Mycoplasma and L-forms: These studies are providing fundamental information relating to the role of *Mycoplasma* and L-forms in human diseases and applicable information for the detection and identification of *Mycoplasma* in cell cultures used for propagation of viruses for vaccines. Tests for lymphocyte cell transformation in which incorporated tritiated thymidine is determined showed a greater response of human lymphocytes to pathogenic beta-hemolytic streptococci than to non-pathogenic alpha-hemolytic streptococci. Cell wall extracts and purified M proteins were better stimulants than the whole bacterial cell. With guinea pig cells, stimulation was limited to lymphocytes from specifically immunized animals.

An inhibitory effect of phytohemagglutinin transformation of human lymphocytes by *Mycoplasma* strains was dependent on the ability of the strain to utilize arginine. Such strains deprived the lymphocytes of an essential amino acid. Excess of arginine prevented the reaction. Strains of *Mycoplasma* that utilize dextrose had no inhibitory.

Tuberculins: Further information has been gained concerning inhibition of macrophages from peritoneal exudate cells from sensitive guinea pigs. The test reveals that the tuberculin-active carbohydrate as well as the specific protein faction detects tuberculin sensitivity of the guinea pig. Clinical studies in progress should reveal whether or not the carbohydrate is an effective diagnostic substance.

With the decrease of tuberculosis in the United States and the relative increase in persons sensitized to atypical mycobacteria, there is a need for skin test substances that would specifically indicate the sensitizing agent. Commercial tuberculins have cross-reactivity which results in positive "tuberculin" reactions which have led to unwarranted therapy for tuberculosis. Tuberculins prepared by the National Communicable Disease Center of the Public Health Service from atypical mycobacteria, when used in low dosage, do tend to differentiate the sensitizing organisms. Tuberculins prepared from seven species in the Laboratory of Bacterial Products invariably showed greatest reactivity in the homologous sensitized guinea pigs. There was considerable variation in cross-reactivity. With the application of the new technique, blast cell transformation of lymphocyte, the reaction of cells from the lymph nodes of guinea pigs and the peripheral blood of sensitive humans correlated with the skin tests. This test, in the experimental stages, shows promise of being a good laboratory tool for the assay of potency and specificity of tuberculins.

Poison ivy and related extracts: This study, which has been in progress for a number of years, has revealed much fundamental information about the mechanism of catechol sensitization and the relationship of the chemical structure to the ability to induce delayed contact sensitivity. However, the ultimate goal of whether or not the extracts of Rhus plants are capable of producing desensitization has not been attained. The first clinical phase showed that poison ivy extract, devoid of urushiol, was ineffective. Pure

urushiol is now on test in two clinics. Information about the distribution of pentadecylcatechol (obtained by the breakdown of urushiol) in the guinea pig has been obtained by use of radioactive compounds and the ratio of haptene to protein in the conjugates determines the ability to induce immunologic responses.

Allergenic extracts: The quantity of antigen E in commercial ragweed correlated in many, but not all instances, with the protein nitrogen unitage (PNU) which is generally used by manufacturers to indicate strength of an extract. When skin tested in man, the extracts having the common PNU values fell into two groups. One was 10 times more active than the other. The difference is being investigated. Investigations of other pollens are in progress. Ether extracts containing both lipids and proteins have induced delayed sensitivity in guinea pigs. The possibility of using the gel-diffusion technique to identify pollen extracts in mixtures and also to estimate the amount of antigen present is being studied.

Cholera vaccine and toxins: Collaboration with the Pakistan-SEATO Cholera Research Laboratory has continued in the selection and assay of vaccines used in the controlled field trials. In this endemic area the protective response of the adult has been like that to a booster injection, and seems to persist up to two years. However, with the child under 5 years, the most susceptible age group, response is of shorter duration and results to-date reflect potency as measured in the mouse potency assay. A single dose of a particular lot of vaccine in the 1963 and 1964 trials afforded 75-80% protection in this age group, while a vaccine of lesser potency afforded only 24% in the 1966 trial. Two doses spaced four weeks apart were more effective. Results apply only to the Inaba serotype as this serotype has been the prevailing infecting organism. In the 1968 trial monoserotype vaccines will be used to determine if there is cross protective action. Protection has been antibacterial in nature as the vaccines have contained no antitoxin stimulating property.

Interest in the role of cholera "toxin" has remained high. Formolinized toxoid has been found to be three to five times more antigenic in guinea pigs and rabbits than heat-inactivated toxin. This formolinized-toxoid is capable of inhibiting fluid flux in the dog following intraintestinal challenge with cholera toxin (collaborative study with Johns Hopkins School of Medicine). Through contracts large quantities of "toxins" are being procured and analyzed (NIAID). The Laboratory of Bacterial Products has provided a project officer for four of the contracts.

Pertussis vaccine: During this year particular emphasis has been directed towards laboratory assays of the reactive factors of pertussis vaccine. A special investigation on the reactivity of pertussis vaccine is in progress. The Laboratory and the licensed manufacturers are cooperating in the assay of two dried vaccines by a number of procedures. The same vaccines are to be used in clinical studies. It is hoped that at least some of the laboratory assays will reflect clinical reactivity.

Serotyping of *Bordetella pertussis* has provided information on the prevailing serotype strains in the United States. The analyses of the strains

used for vaccine production showed that some had lost one or two of their original antigenic factors. This, combined with information elsewhere, indicates a loss in protective antigenicity of the strain. There continues to be an occasional report of an isolated epidemic of pertussis. Incidence of pertussis has been greater in the age group 15 years or older and has been related to time interval since vaccination. So far, protective activity of vaccines in the United States has not been related to serotype of the vaccines.

Typhoid vaccine: The U. S. prescribed potency assay for typhoid vaccine seems to reflect efficacy in man in the case of acetone and heat-killed phenolized vaccines but not of alcoholized vaccines. The latter is attributed to a high **Vi** antigen content. Vi antigen from other species of bacteria, as well as from *Salmonella typhosa*, protects mice against *S. typhosa* infection while for man it is apparently not a protective antigen. Efforts were directed toward assay methods that will reflect human efficacy for all types of typhoid vaccine. Three strains of mice differed in the formation of Vi antibody following whole cell vaccinations but did not differ in relative protective response. With purified Vi antigen the mouse strains differed both in antibody formation and protective activity. It remains to be determined whether use of a particular strain of mouse will solve the problem of potency assay.

Mice for laboratory assays: Earlier studies have shown that results of potency assays are greatly influenced by the ability of the mouse to be sensitized to histamine by pertussis vaccine and the results of the toxicity tests by the intestinal microbial flora of the mouse. In cooperation with the Division of Research Services, a selective breeding project was initiated with a view to obtaining a strain of mouse that can be uniformly rendered sensitive to histamine and have a high degree of uniformity in immune response to antigens.

Currently many animal breeders are marketing mice with a defined bacterial flora. These mice as well as germ-free mice are many times less susceptible to the toxicity of pertussis vaccine (weight gain test) than conventional mice. Hence, they are not suitable for this toxicity test. However, in preliminary protection test experiments these mice have been found to respond in a manner similar to the conventional mouse.

LABORATORY OF BIOPHYSICS AND BIOCHEMISTRY (LBB)

Virus detection: Major techniques under development this year for detecting latent adventitious viruses are: (a) electron microscopy, (b) fluorescence microscopy, (c) precipitin reactions and (d) cytochemical reactions. Modification of these techniques have been designed to detect small concentrations of viruses or viral antigens, whether the agents are already known or not. Cell systems studied intensively for the incidence of contaminating viruses include primary canine and primary simian cells. These studies provide new data concerning the incidence of adventitious viruses in vaccine-related cell types, the identity of the agents isolated and the effectiveness of the detection systems as applied at DBS and elsewhere.

Virus characterization: Viruses studied this year included adeno-associated viruses, infectious canine hepatitis virus, rubella virus, monkey hemorrhagic disease virus, and unidentified canine agent (recently isolated) and an unidentified simian agent (also recently isolated). Studies have been designed to correlate the physical and biological properties of these viruses.

Analytical chemistry: Methods for chemical analysis of biological products developed or evaluated by the group included a comparison of electrolytic and gravimetric methods for analysis of moisture in dried products, and determination of mercurial preservatives and alum or aluminum phosphate in biologicals by atomic adsorption spectrophotometry. Additional studies were concerned with the development of a method to measure the amount of silicone lubricant washed from disposable syringes with water, the amount of ether and alcohol remaining in Rickettsial vaccines after manufacture, and a collaborative study with the Laboratory of Bacterial Products concerning the effect of a bacterial enzyme on the fatty acid composition of Arlacel. Members of the analytical chemistry group have also continued to obtain analytical data on a variety of biological products to serve as a reference background for the chemical control of biological products.

LABORATORY OF BLOOD AND BLOOD PRODUCTS (LBBP)

The research program of this laboratory includes studies of the stability of blood products, which yield information needed in developing acceptable storage conditions and dating periods for materials derived from blood. Proteins of the clotting and fibrinolytic systems are targets of active investigation designed to provide a basis for improved control tests for these products. This year, increasing attention has been devoted to the stability, characteristics, preparations, and assay of serum proteins with antibody activity in view of the great interest in immunologically active products derived from blood.

A study directed toward evaluating the physical stability of human immunoglobulin preparations has been continued. Ultracentrifugal measurements to date have indicated that the endogenous proteolytic activity of Immune Serum Globulin (Human) could be greatly reduced, but not abolished, by heating at 45°C. or 50°C. prior to storage.

Similarly, tests performed during the third year of storage of Blood Grouping Sera have revealed no appreciable loss of potency. However, in a parallel study of Anti-Rh Typing Sera, undertaken during the past year, complete loss of potency was detected in some of the sera used for saline, tube tests.

Information has also been obtained regarding the stability of canine blood grouping serum during storage. The antisera originally obtained in connection with NIH programs have been widely distributed to various investigators for use in establishing dog blood donor colonies. The methodology developed during these studies has been made available to researchers wishing

to prepare their own purified antigens and specific antisera. The burro anti-canine gamma globulin has been used in the Laboratory of Biophysics and Biochemistry of DBS in the detection of adventitious agents in cell cultures by immunofluorescence technique.

By continual monitoring of antihemophilic factor (Factor VIII) activity with a thromboplastic generation test, it was found that the original antihemophilic factor reference plasma had lost potency (approximately 20 percent) over a period of 4.5 years at -20°C. A new reference plasma has been obtained, tested, and found to be satisfactory as a reference standard for antihemophilic factor. Since freeze dried cryoprecipitate could be tested satisfactorily by the procedure used for assay of less purified preparations, it is anticipated that a freeze dried cryoprecipitate may also be a useful reference material.

In other studies concerning blood coagulation, the ability of human thrombokinase to convert prothrombin to thrombin was shown to be independent of any residual thrombin content. This converting activity is inhibited by soybean trypsin inhibitor but not by diisopropylphosphorofluoridate. Of the phosphonates tested, none inhibited thrombokinase unless used at very high concentration. Various co-factors (polylysine, calcium alone, or beef serum or phospholipid in the presence of calcium) were found to accelerate the conversion of prothrombin to thrombin by thrombokinase. Tryptic peptide maps of prothrombin and thrombokinase were completely different, showing that the latter molecule cannot be derived from the former. Bacterial thermolysin could activate both prothrombin and prothrombokinase; subtilisin could activate prothrombin.

In a third area of coagulation research, carried out in association with the Laboratory of Clinical Biochemistry, NHI, ε-(γ-Glutamyl) lysine was shown, by direct demonstration of the isolated dipeptide, to be the crosslink in human fibrin polymerized by human fibrin stabilizing factor (Factor XIII). The amount of crosslinking could be diminished by using inhibitors of Factor XIII, or by using fibrinogen which had been purified to render it free of Factor XIII as the starting material for the experiments.

In view of the pressing need for control of the recently licensed Rh_o (D) Immune Globulin (Human), a large portion of the staff's time has been devoted to research concerned with examining this material and developing a suitable assay for it. A preparation has been obtained which can be used as a reference in anti-Rh_o (anti-D) assays. This preparation has been examined by a variety of methods, as have several other preparations. Rh_o (D) positive donors have been identified whose cells are of suitable antigenicity for use in the radioimmunoassay. A radioimmunoassay with its required ancillary procedures (radio-labeling, chromatography, ultracentrifugation, counting, computerization of data, etc.) has been carried out with some success. However, when its demands in terms of time, cost, and staff are compared with the resultant precision, it becomes clear that a more suitable routine assay is desirable.

Studies of the body fluids of laboratory animals, carried out in association with the Laboratory of Biology, NCI, have been continued. Gel filtration of the nondialyzable material in the urine of normal inbred mice yielded three fractions, the first of which contained uromucoid (Tamm-Horsfall mucoprotein), the second of which contained the major urinary protein (MUP) complex, and the third of which was low in nitrogen, high in carbohydrate and had a low sedimentation coefficient (0.5 S). Tryptic mapping of the individual components of the MUP complex, isolated by ion exchange chromatography, indicated that electrophoretically distinguishable components 1 and 2, a genetic variant shown in this laboratory to be under the control of one genetic locus (the Mup-a locus), differed by a single peptide. Components 1 and 3, on the other hand, had a number of peptides in common plus several peptides unique to each. The peptide map of any given component did not differ between sexes or between the strains investigated.

To supplement the research activities of the Division and to yield data bearing on the development of regulatory standards, four research contracts for which members of the Laboratory are senior project officers are in force. Two of these are concerned with the effects of storage conditions on plastic blood containers and two are concerned with the study of the carcinogenic potential of chemicals used in biological preparations.

LABORATORY OF CONTROL ACTIVITIES (LCA)

Smallpox vaccine: Studies associated with problems in the control of smallpox vaccine have been directed toward dried smallpox vaccine intended for administration by the jet gun. Because a portion of the vaccine is injected below the skin by the jet gun, it has been considered advisable to require that smallpox vaccine intended for jet injection be free of viable bacteria. With present vaccine prepared in the skin of calves it is difficult to achieve preparation free of viable bacteria. When present in relatively small numbers in smallpox vaccine, bacteria are difficult to detect. Therefore, studies have been initiated to devise sensitive techniques for determining the level of viable aerobic and anaerobic bacteria in smallpox vaccine. These tests will then be applied to the control testing of the vaccine.

U. S. Standard Tetanus Antitoxin: The supply of the dried "master" used in the preparation of the U. S. Standard Antitoxin is nearly depleted. This dried preparation was adopted in 1907 and has been in use since that date. Five lots of equine tetanus antitoxin and one lot of tetanus immune globulin (Human) are being studied in order to select one having suitable properties for use as a replacement for the master preparation. Work to date indicates that avidity of these preparations varies depending upon the type of toxin used. In addition to this study, testing performed in the Laboratory on lots submitted for release indicates that tetanus immune globulin (Human) differs from tetanus antitoxin (equine) in the nature of the toxin-antitoxin neutralization reaction which occurs. Studies are in progress to investigate these differences. One obvious difference is in the

type of antigen used to produce the antibodies. Horses are hyperimmunized with relatively crude toxin following preliminary doses of toxoid. Human blood donors are hyperimmunized with toxoid of relatively high purity.

Yellow fever virus seed: In collaboration with the Laboratory of Virology and Rickettsiology and the Laboratory of Pathology the safety and antigenicity of RIF-free primary and secondary yellow fever seed virus preparations derived from the RIF-free virus prepared by the Laboratory of Virology and Rickettsiology were studied. These preparations were found to be as safe and antigenic as the present seed by use of laboratory tests recommended by WHO and the U. S. Minimum Requirements for Yellow Fever Vaccine. (Clinical confirmation will be necessary before the seed can be recommended for routine vaccine production.)

Testing of fluid thioglycollate medium: The growth requirements of *Bacteroides ovatus* and *B. volgatus* were compared with those of *Clostridium tetani* and *Clostridium chauvoei* in order to select a nonsporulating anaerobe suitable for use in testing the growth promoting qualities of Fluid Thioglycollate Medium. Selection was based on a comparison of the growth of these organisms in control thioglycollate with that in modified thioglycollate, i.e., media from which one ingredient had been selected. In this study, *B. vulgatus* 8482 (ATCC) proved to be more fastidious in its growth requirements than the other cultures tested.

Antivenin: Research conducted in the Laboratory provided data leading to the licensing of coral snake antivenin in September 1967. A preparation has been obtained through plasmaphoresis of hyperimmunized goats, which is being characterized for use as a standard antiserum for coral snake antivenin. Studies of venom from various species of coral snakes indicate that venoms from a few species are not neutralized by antisera prepared from venom of other species. However, some degree of cross-neutralization occurs among the majority of species. Venoms from various species and subspecies of coral snakes have been characterized by acrylamide disc electrophoresis and agar gel electrophoresis. Preliminary work involving attempts to characterize antivenins by agar gel immunoelectrophoresis has proved promising. The work with disc electrophoresis of venoms suggests that this technique could be used in identifying species and subspecies of coral snakes.

Opacity Standard: The U. S. Opacity Standard used in the standardization of cell concentrations of bacterial vaccines and of bacterial challenge suspensions used in potency tests is composed of finely divided glass particles suspended in water. During use and storage the opacity of the standard gradually lessens due to changes in particle size and adherence of particles to the walls of the container. Another undesirable feature is the difficulty of reproducing the properties exactly from one lot to the next. For example, the present international preparation differs from all previous international preparations as well as from the U. S. standard. This is causing serious problems in laboratories throughout the world. It has been reported that a stable opacity preparation can be prepared by suspending finely divided titanium dioxide in a solid resin. Efforts are being made to determine if such a preparation is feasible for use as the U. S. Opacity Standard.

LABORATORY OF PATHOLOGY (LP)

Much of the effort of the laboratory is now being directed toward research in, and the development of, test systems for the newer attenuated viral vaccines. Thus, programs are underway to study the pathologic alterations induced by wild adenoviruses, rubella virus, and mumps virus in experimental animals so as to evaluate the attenuation of such viruses in live virus vaccines. These studies are being done in collaboration with other laboratories within the Division.

Rubella virus: In collaboration with the Laboratory of Viral Immunology, studies have continued with wild and attenuated rubella virus both in pregnant and non-pregnant rhesus monkeys. The virus can infect these animals and multiply, although no clinical syndrome develops in either the adult or the fetus.

Adenovirus: In collaboration with the Laboratory of Virology and Rickettsiology and the Laboratory of Biophysics and Biochemistry, studies of wild and "vaccine" strains of human adenovirus 4 are in progress. Attempts to infect monkeys appear to be unsuccessful but the virus apparently does cause an inflammatory reaction when placed directly within the central nervous system. This reaction can be found whether crude or purified virus is used. This phenomenon is of great interest and further studies are being undertaken to elucidate the mechanism.

Mumps virus: A similar reaction has been found to occur when mumps virus (wild or attenuated strains) is inoculated into the central nervous system of monkeys. However, the virus does not replicate within the CNS and this reaction does not appear to be related to neurotropism in man. Furthermore, the attenuated (vaccine) strain of mumps virus will not infect monkeys when inoculated parenterally, although an antibody response is obtained. These studies contributed to the promulgation of Additional Standards for Mumps Virus Vaccine, Live, with the subsequent issuance of a license for this product.

Yellow fever vaccine: Yellow Fever Vaccine (17D strain) has also been under study in collaboration with the Laboratory of Virology and Rickettsiology and the Laboratory of Control Activities. The neurovirulence properties of this virus have been found to be stable even when a virus of the avian leucosis complex has been removed from the seed virus pool. Since Yellow Fever is a disease that affects the central nervous system as well as the viscera, and since the vaccine has neurotropic properties for rhesus monkeys, control of the stability of this neutropism at a low level is essential for use of this new and more purified vaccine in man. The immunogenicity of this derivative of the primary seed strain and its stability are being investigated.

Electron microscopy: The Section of Histopathology has expanded its activities in electron microscopy and has developed excellent techniques. Studies of the ultrastructure of the central nervous system are designed to complement previous similar studies using light microscopy. As the Section perfects its techniques for ultrastructural studies, it is expected that collaborative programs using such techniques will increase and expand.

The Section of Histopathology has continued to perform clinical laboratory techniques and has acquired and learned to operate an automatic counter for assaying red and white blood cells. This has enabled the technical staff to perform such procedures with great efficiency.

Demyelinization studies: Studies of the myelinization of the central nervous system of animals has been expanded to include studies of demyelinating disease, such as experimental allergic encephalomyelitis. A study of congenital deformities of the central nervous system of animals has been initated. Such a baseline study is essential to subsequent evaluation of teratogenesis in relationship to the use of biological products.

Marmoset colony: The Section on Animal Testing, in addition to performing neurovirulence tests on all lots of Poliovirus Vaccine, Live, Oral, and selected lots of Poliomyelitis Vaccine, Measles Vaccine, Live, and Mumps Vaccine, Live, has recently established a colony of South American monkeys, namely, marmosets *(Mystax saguinas)*. Interest in this primate has increased greatly recently since it has been found to be susceptible to Rous sarcoma virus and has been thought possibly to be of use in the study of the pathogenesis of infectious hepatitis. In October, 1967, the Animal Test Section obtained and established a colony of 85 marmosets. The purpose of establishing the colony was to develop skills in the handling, care, and breeding of these animals as well as to develop background information concerning the microorganisms harbored by them. Only when these goals have been accomplished, can these animals be considered suitable for experimental studies. The colony is under the direct supervision of a research microbiologist who is a new addition to the staff of the Laboratory and who has established the necessary laboratory facilities to perform such studies. The colony has decreased from 85 to 69 in the six months since it has been established, the primary cause of death being the effects of overwhelming parasitic infestation. The Laboratory has been fortunate in being able to consult with the veterinarians in charge of the Primate Quarantine Unit, Laboratory Aids Branch, NIH, as to the care of the marmosets. This has been most helpful since the Laboratory lost its own veterinarian in October, 1967. It is hoped that, by the time the colony of marmosets is a year old, a complete microbiological profile will be established, methods of handling will be improved, the diet will be stabilized so that weight gain will be adequate, and a nucleus of laboratory-bred marmosets will be available for further studies.

Viral oncogenesis: Research efforts in the general field of the pathogenesis of infectious disease with particular emphasis on oncogenic viruses is underway. The pathogenesis of the particular tumors induced, the effect of altered immunologic mechanisms, and the interaction of these viruses on each other are being studied. Attempts to prevent tumors by specialized immunization procedures are in progress.

LABORATORY OF VIRAL IMMUNOLOGY (LVI)

Rubella: This has been a year of considerable progress in the rubella field. The Laboratory has maintained its position at the center of the international program to develop a vaccine to control and perhaps eradiate this

important cause of crippling birth defects. The HPV-77 strain of rubella virus has been used by pharmaceutical companies in preparing approximately two hundred thousand doses of experimental vaccine. At present these preparations have been field tested to several thousand individuals and by the end of the current fiscal year the number inoculated should reach 20,000. Rubella vaccine will probably become commercially available during calendar year 1969.

Use of the rapid blood test (HI procedure) developed by the Laboratory in 1966 to assay vaccine-induced immunity has played a key role in advancing the date of expected completion of vaccine development from the early 1970's to 1969. This acceleration of the program should substantially reduce vaccine development costs. Because of their obvious commercial value, patent applications covering the vaccine and the HI test were filed in 1966.

In recent months the Laboratory resolved several fundamental questions by developing and studying highly modified strains of rubella virus and by producing and clinically testing experimental vaccines prepared from these viruses. It was shown that "overattenuated" virus was incapable of inducing a satisfactory immune response in man. Moreover, replication of the vaccine strain of virus in the respiratory tracts of recipients appeared to be essential to the production of protective antibodies. These data permitted determination of the degree of virus modification needed for vaccine production.

Viruses replicate only in living cells and several options exist in the selection of animal species as donors of tissues for cell cultures used in rubella vaccine preparation. For evaluation of the advantages and disadvantages of these systems, experimental live rubella virus vaccines were produced by the Laboratory in primary monkey kidney and chick embryo fibroblast cell cultures by two pharmaceutical research teams. These experimental preparations, eight in all, were subjected to comparative clinical tests by members of the laboratory. The results indicated that all of the vaccines were free of harmful effects. The attenuated viruses grown in monkey, dog and duck cells were fully immunogenic whereas chick embryo adaptation of the virus was shown to have resulted in a significant reduction in vaccine immunogenicity. From such studies it becomes possible to identify the optimum tissue for use in final vaccine development.

Initiation of the first experimental live rubella vaccine study in the open population was made by members of the Laboratory last fall. All earlier trials had been performed in institutions where rigid quarantine measures could be enforced. The protocol for vaccine evaluation in family groups has since been used by others for extensive open population field testing.

Important preparations have been made looking towards biologics control procedures relating to rubella vaccines by: standardization and distribution of a reference serum, development of a sensitive immunofluorescent plaque technique for rapid assay of vaccine potency and determination of the value of new types of cell cultures for rubella virus isolation. Basic investigations of the relationship between hemagglutinating particles and infectious virus have been completed.

Mumps: A live virus vaccine for mumps became commercially available in January 1968. Anticipating this event, the Laboratory has conducted a mumps research program for more than three years. Clinical trials by the group with the Jeryl Lynn strain established that the attenuated virus cannot be detected in the saliva, blood or urine of vaccine recipients. Use of the vaccine in these studies was not associated with adverse reactions in either adults or children. An ongoing investigation designed to determine the duration of vaccine-induced immunity has shown that the level of protective antibodies appears to be unchanged one year after immunization. These data were made particularly meaningful by the inclusion of a control group that by chance experienced natural mumps at approximately the same time that the test group was vaccinated.

The development of a laboratory "marker" system capable of distinguishing attenuated from virulent mumps virus and of a vaccine potency assay test further insured an easy transition from the phase of developmental research to commercial use of the new product without delay.

Other investigations have been concerned with central nervous system infections caused by mumps virus. In this work hydrocephalus could be produced in newborn hamsters with regularity by the direct intracerebral inoculation of either virulent or attenuated virus. This experimental system may be of particular note because of the growing scientific interest in the role of viruses in chronic disease states.

Smallpox: A discussion of smallpox immunization might seem inappropriate in describing modern vaccine research. Such is not the case. Although endemic in areas of Asia, Africa and South America, smallpox no longer exists in North America. Nevertheless, the continued need to immunize all American children results in some 12 vaccine-associated deaths per year and many thousands of other complications.

Viewing these problems the Laboratory has been interested in: (1) finding more efficient methods to immunize the people of the developing nations at direct risk to smallpox and (2) searching for safer ways to perform primary vaccination of populations in countries not exposed to smallpox.

As an outgrowth of research by the Laboratory, the first combined live measles-smallpox vaccine was licensed this year. This product which is given by jet injection should be particularly useful in overseas technical assistance programs.

The logical approach to the second item mentioned appears to be in developing a more attenuated vaccinia virus or a method of immunization less prone to induce undesirable reactions. Perhaps primary vaccination of the child could have sensitization rather than protective immunization as its goal. Infants sensitized to the virus might then be revaccinated with more immunogenic material but at considerably less risk.

Studies of the pathogenesis of vaccinia infections in monkeys provided data that guided the direction of a clinical trial using small doses of virus inoculated subcutaneously. The results were promising.

Infectious mononucleosis and herpes-like virus infections: Many groups have worked with a herpes-like virus present in certain strains of cells cultured from cases of Burkitt's lymphoma. The Laboratory's Section on Viral Genetics found that cultured leucocytes from several healthy donors in this country contained similar herpes-like virus particles: Using sensitive serologic tests it was possible to show that antibodies to this agent are widespread in the population and bear no apparent relationship to the presence or absence of antibodies for herpes virus of man, i.e., herpes virus hominis (simplex). Related antibodies were very commonly found in sera of subhuman primates (except baboons) but not in specimens from common species of lower animals.

Recently, reports have appeared to give evidence that herpes-like viruses caused infectious mononucleosis in man. Using sera provided by the group at Yale University, investigators in this Laboratory were able to confirm the serological association of herpes-like virus infections and infectious mononucleosis.

This area of investigation is especially exciting because of the theoretical possibility that infectious mononucleosis could be a sub-leukemic manifestation of infection caused by a human leukemia virus.

Viral hepatitis: A continuing effort is being directed toward the elucidation of the etiology of viral hepatitis. Investigators in the Section on Immunology identified two agents recovered by workers at the Johns Hopkins University as reoviruses. Studies in the Laboratory also provided results indicating that these reoviruses were not related to human hepatitis.

New approaches such as fluorescent techniques and cell fusion methods are being applied to specimens from hepatitis cases. In a prospective study being conducted in cooperation with another institute, specimens are collected periodically from surgical patients who have received blood transfusions.

If and when the etiologic agents of viral hepatitis are cultured in the laboratory a major effort to develop vaccines can be expected.

Diseases caused by genetic defects: The Laboratory has maintained a small program concerned with cell culture studies of diseases caused by genetic defects. Diploid cells derived from selected tissues and maintained by serial propagation in culture retain certain metabolic characteristics of the parent tissue. Consequently, appropriate studies of these cells can provide information identifying a specific heritable disease or genetically determined predisease state of the donor. A novel use of this technique now being explored involves culture of cells which have been obtained by amniocentesis to determine genetic flaws in the fetus during early gestation.

Vaccine-related cell culture studies: With the need to obtain detailed data regarding several types of animal cells potentially important in the study or production of vaccines, it was necessary to produce a variety of special cell cultures in the Laboratory.

The Section on Immunology produced the equivalent of 30,000 tubes of avian, leucosis-free, primary chick embryo cell cultures in supporting the rubella research program. These cells were from eggs of several ages, from both whole embryo and selected embryo tissues and grown under varied environmental conditions. Such exacting requirements were necessary in determining the factors that would permit adaption of rubella virus to replication in chick cells and in the later production of experimental chick embryo propagated rubella vaccines for clinical trial.

Each of the cell production efforts represents a special research project. Another example concerns the 7,000 duck embryo cultures (tube equivalents) prepared by the Section. A pharmaceutical company proposes to manufacture a major live virus vaccine (rubella) in primary duck embryo cultures. Consequently, detailed information relating to chick cells was needed. Investigators produced and tested duck cultures on a weekly basis to obtain the necessary answers. Trial lots of experimental vaccine were prepared in duck embryo cells and virus growth characteristics determined. Primary cell cultures and duck fibroblasts maintained by serial transfer for up to 15 passages were studied for extraneous agents using cytopathic tests, the interference procedure and other appropriate techniques.

LABORATORY OF VIROLOGY AND RICKETTSIOLOGY (LVR)

Influenza vaccines: During the year the influenza vaccine control program was re-examined by the newly organized Respirovirus Unit. Several areas were defined as needing attention, especially in the performance of technical procedures of CCA determination and mouse potency tests. These procedures are now being standardized and a collaborative study between DBS and manufacturers' laboratories employing these methods is in progress. Two large studies on volunteers inoculated with influenza vaccines of current virus strain composition were performed and the results are being analyzed to compare the immune responses elicited in man and the mouse.

Yellow fever vaccine: The standard yellow fever vaccine seed has been purified by simple membrane filtration. The resulting primary and secondary vaccine virus seeds were shown to be indistinguishable in animal experiments from the standard RIF-virus-contaminated yellow fever vaccine. Human trials are being organized. In the course of these studies, a plaque neutralization test was developed; it seems to have many practical and theoretical advantages over the currently used yellow fever mouse neutralization test.

Smallpox vaccine: It has been shown that electron microscopic counting of vaccinia virus particles extracted from smallpox vaccines may be useful in assaying vaccine potency. In the course of this study, reactivation of heated poxvirus, using UV-inactivated virus as the reactivating virus, has been accomplished--an observation of considerable importance to the understanding of the biology of poxviruses.

Vaccines produced in eggs: Production of egg vaccines has been complicated by the necessity of surveying the avian flocks for the potentially troublesome

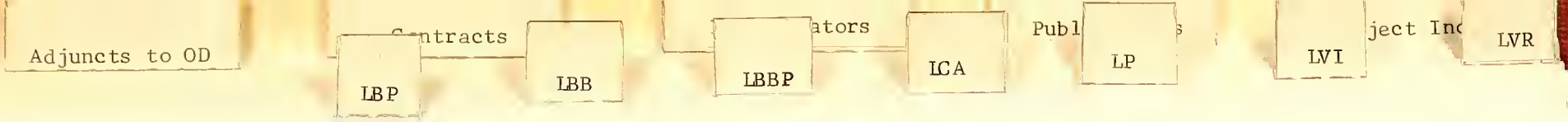
microbial infections. This involves bleeding the birds, and examining the serum using a variety of serological procedures. Some of these procedures are cumbersome, relatively insensitive or non-specific. By combining the methodology of indirect immunofluorescence with the use of egg yolk extract (in place of bird serum), a simple, reliable, and inexpensive procedure has been worked out. It seems to be useful in detecting avian antibody against major groups of microbial antigens, and has distinct practical advantages for certain antibody tests.

Rickettsial vaccines: Several problems of practical and fundamental importance in relation to rickettsial vaccines are being investigated. Work continues on the examination of the usefulness of complement fixation tests in assaying the potency of typhus vaccines. Fundamental studies on the relationships among rickettsiae and on their relationship with host cells are in progress, employing biochemical and biological procedures, including DNA base ratio determination and homology trials. In the experiments using *Rickettsia quintana* as a model rickettsia, important nutritional requirements of the organism in cell culture are being defined.

Hemorrhagic fevers: The importance of the continuing studies on simian hemorrhagic fever has been underscored by the isolation of a virus from rhesus monkeys with hemorrhagic disease at the Sukhumi, USSR, primate colony. The Sukhumi virus was found to be indistinguishable from the Bethesda virus isolated and reported on last year. Recently another outbreak, apparently due to the same agent, occurred at the National Center for Primate Biology (Davis, California); on request the laboratory assisted with the investigation. Other collaborative studies on the pathogenesis of hemorrhagic fevers suggest that, in common with certain other viral infections, an underlying mechanism may lie in the now well defined clinical-pathological syndrome of "consumptive coagulopathy". This suggest that paradoxically anticoagulants might be useful in the treatment of viral hemorrhagic fevers.

Slow, chronic and latent infections and diseases: A new class of unusual agents causing remarkable disease in man (kuru) and animals (scrapie, visna) has been defined. Because of the potentially great importance of some of these agents as possible contaminants of biologics products, a project has been initiated in this area. This is being coordinated with work on the auto-immune mechanisms that become involved through the action of live or inactivated virus antigens.

Cell cultures: A many faceted program of research and development in connection with the production of cell cultures for the Division is actively pursued. As a result, many practical improvements have been introduced into routine cultivation of cells from usual and unusual sources. Increasingly greater attention is being focused on the biology of cultured cells, including certain immunologic aspects.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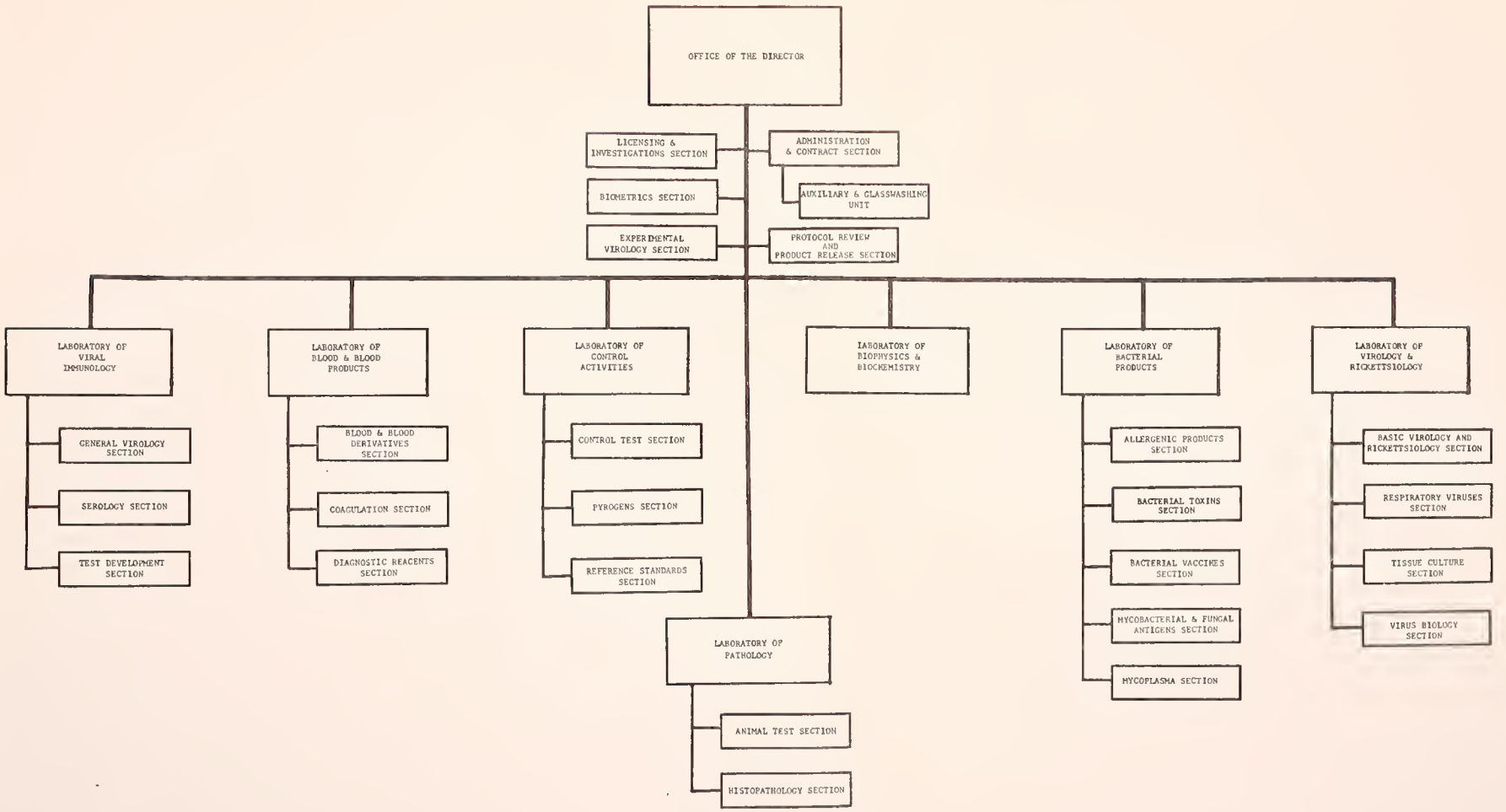

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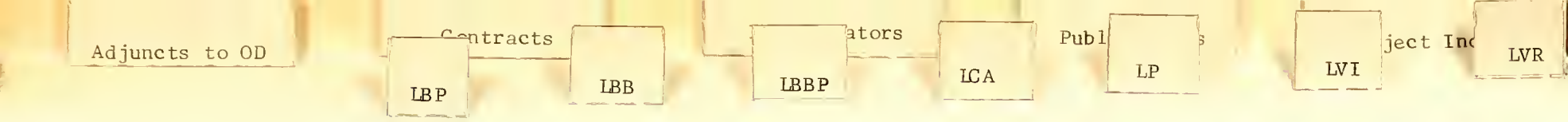
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Annual Report of the
Laboratory of Bacterial Products
Division of Biologics Standards
July 1, 1967 through June 30, 1968

Newer developments have indicated a need for the Laboratory of Bacterial Products to increase its research activities along certain lines as well as to continue research directed toward the old line bacterial vaccines and allergenic products. Provisions for potential increase in work on Mycoplasma and tuberculosis vaccines were made by dividing the responsibilities of two sections and creating two new sections. Watch is being kept on the developments of the Vaccine Development Board (NIAID) on the immunizing antigens, pneumococcus polysaccharide, streptococcus M protein and meningococcus vaccine, in order to be prepared to initiate development of technical standards when there is likelihood of a licensed product. The expansion of the program has been undertaken without additional staff, although such would be very helpful. There is particular need for an enzymologist to study the effect of enzymes on reactivity and stability of potency of products and adjuvants.

The special competence of the staff is recognized by invitations to lecture, serve on scientific committees and study groups, etc. The staff actively participates in the regulatory functions of the Division, in cooperative intramural, national and international projects and in collaborative (contract) programs of the Division and other Institutes.

1. Research Activities

Tetanus Studies. The cooperative study on the prevention of neonatal tetanus in New Guinea by injection of various types of tetanus toxoid preparations is nearing completion with the final collections of sera to determine duration of antitoxin levels and booster response to adsorbed and plain toxoids. The results indicate that women of childbearing age given a primary immunization of two injections of adsorbed toxoid followed by a single booster of adsorbed toxoid at the time of pregnancy would have sufficient antitoxin levels to protect their newborn against tetanus (Proj. No. 12, p. 33). In the next phase of the study to be performed at DBS (Proj. No. 14, p. 37) effort will be directed towards relating the findings to a quantitative potency assay for tetanus toxoids and the assessment of the need to specify a unitage requirement in place of the present minimum potency requirement.

In the study (Proj. No. 13, p. 35) on the separation and characterization of antigens in filtrates of Clostridium tetani an esterase activity detected by the use of Arlacel A, olive oil and peanut oil was found to be enhanced by the addition of 7S gamma globulin. The activity is not related to the level of antitoxin added. The mechanism of the reaction and its significance especially in relation to untoward reactivity of oil-in-water adjuvants remains to be determined.

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Water-in-oil Adjuvants. (Proj. No. 15, p. 39) Earlier work showed that although abscess formation of antigen in water-in-oil adjuvant and Arlacel A paralleled the free fatty acids content, the free fatty acids per se were not responsible for the abscess formation. In recent work it has been shown that the components of oil adjuvants, especially Arlacel A, are capable of inducing cytopathic effect in tissue cell cultures. The degree of the cytopathic effect has correlated well with toxicity measured by the intraperitoneal mouse test. The results were reproducible in six cell line cultures. These early findings indicate that cell cultures will be a valuable tool for measuring the toxicity of oil adjuvant components. Results are being compared with the findings of the various DBS contracts which are directed towards the chemical analyses and the biological and immunological effects of these components.

Mycoplasma and L-forms. These studies are providing fundamental information relating to the role of Mycoplasma and L-forms in human disease and applicable information for the detection and identification of Mycoplasma in cell cultures used for propagation of viruses for vaccines. Tests for lymphocyte cell transformation in which incorporated tritiated thymidine is determined showed a greater response of human lymphocytes to pathogenic beta-hemolytic streptococci than to non-pathogenic alpha-hemolytic streptococci. Cell wall extracts and purified M proteins were better stimulants than the whole bacterial cell. With guinea pig cells, stimulation was limited to lymphocytes from specifically immunized animals. (Proj. No. 16, p. 41.)

An inhibitory effect of phytohemagglutinin transformation of human lymphocytes by Mycoplasma strains was dependent on the ability of the strain to utilize arginine. Such strains deprived the lymphocytes of an essential amino acid. Excess of arginine prevented the reaction. Strains of Mycoplasma that utilize dextrose had no inhibitory action (Proj. No. 17, p. 43.).

Tuberculins. Further information has been gained about the inhibition of macrophages from peritoneal exudate cells from sensitive guinea pigs. The test reveals that the tuberculin active carbohydrate as well as the specific protein faction detects tuberculin sensitivity of the guinea pig. Clinical studies in progress should reveal if the carbohydrate would be an effective diagnostic substance. (Proj. No. 100, p. 16)

With the decrease of tuberculosis in the United States and the relative increase of persons sensitized to atypical mycobacteria, there is increasing need for skin test substances that would specifically indicate the sensitizing agent. Commercial tuberculins have cross-reactivity and positive reactions have led to unwarranted therapy for tuberculosis. Tuberculins prepared by PHS from atypical mycobacteria when used in low dosage do tend to differentiate the sensitizing organisms. Tuberculins prepared from seven species in our laboratory always showed greatest reactivity in the homologous sensitized guinea pigs. There was considerable variation in cross-reactivity. With the application of a new technique, blast cell transformation of lymphocyte, the reaction of cells from the lymph nodes of guinea pigs and the peripheral blood

of sensitive humans correlated with the skin tests. This test, in the experimental stages, shows promise of being a good laboratory tool for the assay of potency and specificity of tuberculins. (Proj. No. 135, p. 19.)

Poison Ivy and related extracts. (Proj. No. 102, p. 22.) This study which has been in progress for a number of years has revealed much fundamental information about the mechanism of catechol sensitization and the relationship of the chemical structure to ability to induce delayed contact sensitivity. However, the ultimate goal of whether or not the extracts of Rhus plants are capable of desensitization has not been attained. The first clinical phase showed that poison ivy extract, devoid of urushiol, was ineffective. Pure urushiol is now on trial in two clinics (Contracts #PH-43-64-885, p. 11 and #PH-43-68-632, p. 15). This year has provided information about the distribution of pentadecylcatechol (obtained by the breakdown of urushiol, Contract #PH-43-64-76, p. 9) in the guinea pig by use of the radioactive compounds and the ratio of haptene to protein on the ability of conjugates to induce immunologic responses.

Allergenic Extracts. The quantity of antigen E in commercial ragweed in many, but not all instances, correlated with the protein nitrogen unit (PNU) which is generally used by manufacturers to indicate strength of an extract. When skin tested in humans, the extracts having the common PNU values fell into two groups. One was 10 times more active than the other (Proj. No. 119, p. 24). The difference is being investigated. Investigations of other pollens is in progress. Ether extracts containing both lipids and proteins have induced delayed sensitivity in guinea pigs. The possibility of using the gel-diffusion technique to identify pollen extracts in mixtures and also to estimate the amount of antigen present is being studied. Development of specific standards for allergenic extracts should be intensified. There are, no doubt, more human beings injected with allergenic products than any other biologic.

In a cooperative project the degree of precision and reproducibility that can be attained in skin testing is being studied. (Proj. No. 134, p. 26)

Cholera vaccine and toxins. (Proj. No. 22, p. 45). Collaboration with the Pakistan-SEATO Cholera Research Laboratory has continued in the selection and assay of vaccines used in the controlled field trials. In this endemic area the protective response of the adult has been like that to a booster injection, and seems to persist up to two years. However, with the child under 5 years, the most susceptible age group, response is of shorter duration and results to-date reflect potency as measured in the mouse potency assay. A single dose of a lot of vaccine in the 1963 and 1964 trials afforded 75-80% protection in this age group, while a vaccine of lesser potency afforded only 24% in the 1966 trial. Two doses spaced four weeks apart were more effective. Results apply only to the Inaba serotype as this serotype has been the prevailing infecting organism. In the 1968 trial monoserotype vaccines will be used to determine if there is cross protective

action. Protection has been antibacterial in nature as the vaccines have contained no antitoxin stimulating property.

Interest in the role of "toxin" has remained high. Formalinized toxoid has been found to be three to five times more antigenic in guinea pigs and rabbits than heat-inactivated toxin. This formalinized-toxoid is capable of inhibiting fluid flux in the dog following intraintestinal challenge with cholera toxin (collaborative study with Johns Hopkins School of Medicine). Through contracts large quantities of "toxins" are being procured and analyzed (NIAID). The Laboratory of Bacterial Products has provided a project officer for four of the contracts.

Pertussis Vaccine. (Proj. No. 104, p. 49.) During this year particular emphasis has been directed towards laboratory assays of the reactive factors of pertussis vaccine. A special investigation on encephalopathy is in progress. The Laboratory and the licensed manufacturers are cooperating in the assay of two dried vaccines by a number of procedures. The same vaccines are to be used in clinical studies. It is hoped that at least some of the laboratory assays will reflect clinical reactivity.

The contract (PH-43-66-552, p. 13) on serotyping of Bordetella pertussis has provided information on the prevailing serotype strains in the United States. The analyses of the vaccine-propagation strains showed that some strains had lost one or two of their original factors. This combined with information elsewhere indicates a loss in protective antigenicity of the strain. There continues to be an occasional report of an isolated epidemic of pertussis. Incidence has been greater in the age group 15 years or older and has been related to time interval since vaccination. So far protective activity of vaccines in the United States has not been related to serotype of the vaccines.

Typhoid Vaccine. (Proj. No. 116, p. 51.) The U. S. prescribed potency assay for typhoid vaccine seems to reflect human efficacy for acetone and heat-killed phenolized vaccines but not alcoholized vaccines. The latter is attributed to a high Vi antigen content. Vi antigen from other species of bacteria as well as from Salmonella typhosa protects mice against S. typhosa infection while for man it is apparently not a protective antigen. Efforts were directed toward assay methods that will reflect for all types of typhoid vaccine human efficacy. Three strains of mice differed in formation of Vi antibody following whole cell vaccination but did not differ in relative protective response. With purified Vi antigen the mouse strains differed both in antibody formation and protective activity. It remains to be determined whether a particular strain of mouse will solve the problem of potency assay.

Mice for Laboratory Assays. Earlier studies have shown that results of potency assays are greatly influenced by the ability of the mouse to be sensitized to histamine by pertussis vaccine and the results of the toxicity tests by the intestinal microbial flora of the mouse. In cooperation with DRS a selective breeding project was initiated to obtain a strain of mouse

that can be uniformly rendered sensitive to histamine (Proj. No. 138, p. 57) with the view that the mouse will have a high degree of uniformity in immune response to antigens.

Currently many animal breeders are marketing mice with a defined bacterial flora. These mice as well as germ-free mice are five times less susceptible to toxicity of pertussis vaccine (weight gain test) than conventional mice. Hence, they are not suitable for this toxicity test. However, in preliminary protection test experiments these mice responded similarly to the conventional mouse. The influence of bacterial flora on the establishment of Vibrio cholerae in rodents is under investigation to obtain a possible clearer understanding of the influence of the intestinal flora on human susceptibility to cholera infections. (Proj. No. 136, p. 54)

2. Control Activities

A. Regulatory Standards

a. Prepared technical documents for anthrax vaccine and tuberculin.

b. Assisted technically in matters pertaining to publication of standards for Pertussis Vaccine and test for Mycoplasma, and proposed rule making for Diphtheria Toxin for the Schick Test Toxin.

B. Potency and safety tests (January 1, 1967 through December 31, 1967)

The Laboratory of Bacterial Products performed tests on eight products as follows:

Tests and protocol reviews

Type of product	Purpose	Type of test: Potency	Type of test: Safety Toxicity	Protocols reviewed
Pertussis Vaccine	Official*	98	175	68
	Special, foreign	41	21	
	Standardization	8	2	
Cholera Vaccine	Coop LCA	8	0	23
	Foreign	4	1	
Pertussis Immune Globulin (Human)	Special	7		4
	Foreign	5		
Anti-*Hemophilus influenzae* serum type b	Official	2		
Haemophilus influenzae typing serum	Official			4
BCG Vaccine	Official	5**		16
			Mycoplasma	
Measles Vaccine	Official		9	108
Mumps Vaccine	Official		7	15
Intramural	Special		35	
Totals		178	250	238

* Tests of sample for release, inspection or license application.
** Viability counts.

C. Activities requiring travel status.

Type of activity	Personnel	Locations	Man days of travel
Annual Inspections	6	72	98
Investigations	0	0	0
Contracts	4	16	18

D. License applications and amendments.

Reviews initiated	32	Person participation	84
Labeling reviewed	45	Person participation	90

E. Other Activities.

The Chief, LBP. Project Officer for the Pakistan-SEATO Cholera Research Laboratory (NIH Research Agreement #196802, OIR)

NIH Cholera Advisory Committee

PSCRL, Member of Panel of Expert Consultants to the Technical Committee

U.S. Pharmacopeia, Member of Panel for revision of Sterility Testing and Sterilization

Guest Lecturer at Howard University

Board of Governors, American Academy of Microbiologists

WHO--Planned and supervised a preparation for the replacement of the International Standard for Pertussis Vaccine

Dr. Harold Baer. Committee on Standardization of Allergens, American College of Allergy

Committee on Specific Therapy, American Academy of Allergy

Education Committee, American Association of Immunologists

With Dr. S. D. Chaparas organized DBS Conference on Evaluation of Procedures for Tuberculin Conference, Feb. 28-29, 1968

Dr. S. D. Chaparas. Faculty Chairman, Microbiology and Immunology, NIH Graduate School

Dr. John C. Feeley. Cholera Panel, U.S.-Japan Cooperative Medical Science Program

Cholera and Parasitology Study Section, DRG

Advisory Editor, Journal of Infectious Diseases

WHO Temporary Advisor, Biological Standardization, for Revised International Requirements for Cholera Vaccine, Geneva, April 22-26, 1968

WHO--Participant in Collaborative Assay of International Reference Preparations for Cholera Agglutinating Sera

WHO--5-year appointment as member of Expert Advisory Panel on Bacterial Diseases (Cholera)

Project Officer for three NIAID contracts pertaining to cholera toxins

Staff members have audited conferences, workshops and given lectures.

3. Collaborative (Contract) Activities

Two staff members served as Project Officers and two as Assistant Project Officers for four contracts that are an extension of the LBP research program. The contracts are concerned with the preparation of and synthesis of poison ivy skin reactive substances (Contract #PH-43-64-76 p. 9), the clinical potency of Rhus extract and desensitization (Contracts #PH-43-64-885 p. 11 and #PH-43-68-632 p. 15) and serotyping of Bordetella pertussis cultures (Contract #PH-43-66-552 p. 13). The latter contract was completed. Two members served as Assistant Project Officers on several other DBS contracts.

CONTRACT NARRATIVES
LABORATORY OF BACTERIAL PRODUCTS
DIVISION OF BIOLOGICS STANDARDS
July 1, 1967 through June 30, 1968

Allergenic Products Section

COLUMBIA UNIVERSITY (PH-43-64-76)

Title: Preparation of Poison Ivy and Poison Oak Extracts and the Synthesis of Substances related to the Active Principles

Contractor's Project Director: Prof. Charles R. Dawson

Project Officer (DBS): Harold Baer, Ph. D.

Assistant Project Officer (DBS): Henry Godfrey, Ph. D.

Objectives: (1) To obtain the active principles of poison ivy, poison oak, and poison sumac. (2) To determine whether these substances are present in commercial preparations. (3) To synthesize the active principles and related substances.

Methods Employed: Various isolation, synthetic, and analytical methods are employed.

Major Findings: 1. Sufficient pure urushiol from poison ivy and poison oak was prepared to permit its use in clinical studies; it is anticipated that poison sumac will be studied in the near future. 2. Various new synthetic catechols were prepared to enable studies on the relationship of chemical structure to delayed contact sensitivity. 3. Urushiol, the active ingredient of poison ivy is a mixture of 4 closely related catechols. The 4 catechols have been separated, and supplied in pure form to DBS, and will be used for animal and human studies. 4. Thin layer chromatographic and chemical methods of assay for the catechol content of poison ivy extract have been developed. 5. Enzymes are present in the poison ivy plant that can alter the active principle in various ways. A start has been made on the study of these enzymes.

Significance to DBS and Bio-Medical Research: This project will provide information on a group of licensed products, Poison Ivy Extract, Poison Oak Extract, and, eventually, Poison Sumac Extract, and related products that are potentially subject to license, i.e., Cashew Nut Oil Extract. The chemical composition of the active ingredients will be determined, then the degree of immunologic relationship will be examined to determine whether they might be used interchangeably.

The synthetic substances will be used to examine the relationship of chemical structure to ability of catechols to sensitize and to determine whether some of them may be substituted for the naturally occurring catechols for diagnosis or treatment of poison ivy, oak, or sumac sensitivity.

Proposed Course of Project: To continue until objectives are reached. The current contract will expire December 1968.

Date Contract Initiated: November 18, 1963

Current Annual Level: $40,000

CONTRACT NARRATIVES
LABORATORY OF BACTERIAL PRODUCTS
DIVISION OF BIOLOGICS STANDARDS
July 1, 1967 through June 30, 1968

Allergenic Products Section

ORENTREICH RESEARCH CORPORATION (PH-43-64-885)

Title: Determination of Potency and Efficacy of Urushiol, the Active Ingredient of Poison Ivy Extract

Contractor's Project Director: Robert Auerbach, M. D.

Project Officer (DBS): Harold Baer, Ph. D.

Assistant Project Officer (DBS): Henry Godfrey, M. D.

Objectives: (1) To determine whether poison ivy extract or the active principles can hyposensitize poison ivy sensitive humans. (2) To ascertain whether the administration of these extracts to humans causes any toxic symptoms.

Methods Employed: The pure urushiol was prepared from poison ivy leaves by Prof. Charles R. Dawson at Columbia University under Contract (PH-43-64-76) and was prepared for clinical use by the Pharmaceutical Development Service of NIH. A commercially-obtained poison ivy extract was standardized at DBS by guinea pig potency assay and then supplied to the contractor. The contractor administered these materials parenterally and orally. Skin tests prior to and during treatment were used to assess the efficacy. Blood and urine samples were taken at regular intervals for laboratory examination to aid in determining whether any toxic effects were found.

Major Findings: The purified material has not been administered for a sufficient period of time to obtain useful data. Previously it was shown that the commercial extract had little if any desensitizing effect at the dosage levels given. The administration of these materials in the dosage employed in this study resulted in no evidence of toxicity.

Significance to DBS and Bio-Medical Research: It is frequently stated but not thoroughly established that the administration of poison ivy extracts may reduce or eliminate sensitivity of humans to poison ivy. An earlier study carried out by the contractor using crude poison ivy extract failed to demonstrate a clear cut efficacy. If the purified active components of poison ivy are more effective this could lead to new standards for products of this type.

If hyposensitization is achieved, it may be possible to study the mechanism

thereby achieving a better understanding of delayed contact sensitivity.

Proposed Course of Project: To continue treatment until it is determined that the product is or is not effective.

Date Contract Initiated: May 26, 1964

Current Annual Level: $4,000

CONTRACT NARRATIVES
LABORATORY OF BACTERIAL PRODUCTS
DIVISION OF BIOLOGICS STANDARDS
July 1, 1967 through June 30, 1968

Bacterial Vaccines Section

MICHIGAN DEPARTMENT OF HEALTH (PH-43-66-552)

Title: Serotyping of *Bordetella pertussis* cultures

Contractor's Project Director: Grace Eldering, Sc. D.

Project Officer (DBS): Margaret Pittman, Ph. D.

Assistant Project Officer (DBS): John C. Feeley, Ph. D.

Objectives: To determine the serotypes of *B. pertussis* strains (1) currently used for vaccine production by U. S. licensed manufacturers and (2) being isolated from cases of whooping cough, and to prepare and supply to DBS reference typing sera.

Methods Employed: Manufacturers' strains labeled by code number were serotyped, also strains collected from various parts of the United States were typed. Rabbits were immunized with type strains and the sera adsorbed to assure the type specificity of each serum. The sera were dispensed in vials, freeze-dried, labeled and delivered to DBS.

Major Findings: (1) Reference typing sera were prepared and are available on request from the Division of Biologics Standards for use relating to the manufacturing of pertussis vaccine. (2) Seventy-two *B. pertussis* cultures used by manufacturers to prepare vaccine were serotyped. There were eleven different combinations of factors; 14 of the cultures were serotype 1.3.6; 13 were 1.2.3.5.6; 13 were 1.2.4; and 12 were 1. The other combinations represented by one or more cultures were: 1.3; 1.2.6; 1.2.3.4; 1.2.3.6; 1.2.5.6; 1.2.3.4.6; and 1.2.4.5.6. Thirty-four of the cultures were not agglutinated by factor 3 antiserum. Several manufacturers supplied the same strains. Some of these showed a loss of one or more antigens and a concomitant reduction in agglutinability in a broad spectrum antiserum. With a change in serotype and agglutinability, there is reason to consider that protective activity of the strain might be affected, also. (3) One hundred sixty-three cultures isolated during 1966 and 1967 in eight different areas of the United States and Canada were found to have eight different patterns. The most common serotype was 1.3.6, comprising 82 percent of the cultures.

Significance to DBS Program and Bio-Medical Research: With knowledge about the serotype of production strains and of strains currently causing whooping

cough in the United States, and the availability of typing sera, both the manufacturers and the DBS can better assess the significance of serotypes from a prophylactic standpoint. The typing sera will provide a tool for surveillance of production cultures for loss of serotype antigens which a recent publication indicates loss in protective efficiency.

Proposed Course of Project: The project was completed December 5, 1967 and the final report was forwarded on the same day.

Date Contract Initiated: June 6, 1966.

Current Annual Level: $21,900. Contract was for one year but due to delay in drying of the sera, contract was extended six months without additional funds.

CONTRACT NARRATIVES
LABORATORY OF BACTERIAL PRODUCTS
DIVISION OF BIOLOGICS STANDARDS
July 1, 1967 through June 30, 1968

Allergenic Products Section

UNIVERSITY OF CALIFORNIA MEDICAL SCHOOL (PH-43-68-632)

Title: Determination of Potency and Efficacy of the Active Ingredient of Poison Oak Extract by Clinical Studies

Contractor's Project Director: William B. Epstein, M. D.

Project Officer (DBS): Harold Baer, Ph. D.

Assistant Project Officer (DBS): Henry Godfrey, M. D.

Objectives: To determine whether the pure active principle of poison oak can hyposensitize individuals sensitive to poison oak.

Methods Employed: The active principle was isolated from fresh leaves under Contract (PH-43-64-76) at Columbia University and was prepared for clinical use by the Pharmaceutical Development Service of the NIH. The contractor will administer this material to sensitive patients. Skin tests prior to and during treatment will be used to assess efficacy. Blood and urine samples will be taken at regular intervals for laboratory examination to aid in determining whether any toxic effects can be found.

Major Findings: The contract has not been in effect for a sufficient time to obtain useful results.

Significance to DBS and Bio-Medical Research: It has been often stated, but not scientifically established, that the administration of poison oak extracts may reduce or eliminate the sensitivity of sensitive individuals. Since Poison Oak Extracts are subject to license, it would be of value to DBS to determine if they possessed efficacy. If efficacy is established, it may be possible to establish useful dosage schedules, dosage sizes and the duration of the effect. It may also be possible to obtain information relating to the mechanism thus achieving a greater understanding of delayed contact sensitivity.

Proposed Course of Project: The treatment will be continued until it is determined that patients are or are not hyposensitized.

Date Contract Initiated: January 15, 1968

Current Annual Level: $21,910

Serial No. DBS 100
1. Bacterial Products
2. Allergenic Products
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Composition, Immunology, and Specificity of Tuberculin

Previous Serial Number: SAME

Principal Investigators: Harold Baer, Ph. D.
S. D. Chaparas, Ph. D.
H. P. Godfrey, M. D.

Other Investigators: Sheldon M. Wolff, M. D. (NIAID)
Richard K. Root, M. D. (NIAID)
Joseph Gallelli, Ph. D. (CC)

Cooperating Units: Laboratory of Clinical Investigation, NIAID
Pharmaceutical Development Service, CC

Man Years

Total:	1.7
Professional:	.8
Other:	.9

Project Description:

Objectives: The overall objective is to isolate the specific skin reactive substance(s) of tuberculin, to characterize its chemical and immunological properties and to evaluate the validity of the currently used potency assay for tuberculins with a view to the development of more specific tuberculins for diagnosis of mycobacterial infections and to improve standards for tuberculins.

The project is divided into a series of subprojects as follows: (1) To study methods of inducing and measuring delayed sensitivity and antibody to the carbohydrate and protein components of BCG culture filtrate; (2) to investigate the influence of tuberculin fractions on cell cultures obtained from sensitive animals; (3) To review and evaluate the control testing procedures in the assay of tuberculin preparations.

Methods Employed: Guinea pigs were immunized with heat killed mycobacteria in water-in-oil emulsions. Delayed hypersensitivity was studied in vivo by

skin testing sensitive animals or in vitro by the inhibition of migration of their cells. Fractionation and analysis was accomplished by physical, chemical and immunological procedures.

The carbohydrate fraction which exhibited skin reactivity in guinea pigs was skin tested on human beings.

Major Findings: Subproject 1: Tuberculin active carbohydrate (H. Baer, Chaparas, Wolff, Root). This material is known to be skin-reactive in guinea pigs. Tests for reactivity in the skin of sensitive humans are in progress.

Subproject 2: Suppression of tuberculin activity: This project, completed last year, has been published.

Subproject 3: Tuberculins from atypical mycobacteria: Project transferred to No. 135.

Subproject 4: Skin activity of fractions discarded in the manufacture of PPD. This project was discontinued.

Subproject 5: Inhibition of Cell Migration (Godfrey & Baer). The polysaccharide fraction has been shown to inhibit the migration of macrophages from peritoneal exudate cells from suitably sensitive guinea pigs.

Significance to Bio-Medical Research and the Program of the Division: These studies have revealed new information concerning the composition of tuberculin. It has been demonstrated that there are at least two specificities present in tuberculin sensitive animals which could be one of the factors responsible for variations in specificity of different preparations of tuberculin.

The inhibition of cell migration by the carbohydrate is further evidence that this substance does, in fact, induce a delayed response in guinea pigs.

Studies on the suppression phenomenon have provided information that will permit an increase in reproducibility and reliability of the guinea pig potency assay of tuberculin.

Proposed Course of Project: Studies will be continued on the in vitro inhibition of cell migration by both carbohydrate and protein fractions of tuberculin.

The chemical composition of the tuberculin active carbohydrate is under investigation and a comparison of the carbohydrate and protein fractions in tuberculin sensitive humans will be completed.

Honors and Awards: None

Publications:

Baer, H., and Kolb, R. W.: Suppression of the Tuberculin Reaction by High Concentrations of Tuberculin and the Relationship of this Phenomenon to the Potency Assay of Tuberculin. J. Immunol. 98: 1015-1019, May 1967. (Reported as "In Press" in 1967.)

Serial No. DBS 135
1. Bacterial Products
2. Allergenic Products
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Specificity and Hypersensitivity of Tuberculins

Previous Serial Number: 100 part III

Principal Investigator: Sotiros D. Chaparas, Ph. D.

Other Investigators: Mrs. Sally Hedrick
Paul Chretien, M. D. (NCI)
John D. Harrah, M. D. (NCI)
Robert Bowser, Ph. D. (AFFRI)
John N. Sheagren, M. D. (NIAID)
Anthony N. Demeo, M. D. (CC)
Lawrance R. Draper, Ph. D. (NCI)
Estes Eppele, D.V.M. (USDA)

Cooperating Units: National Cancer Institute
National Institute of Allergy & Infectious Diseases
Clinical Center - Employee Health Service
Armed Forces Radiobiological Institute
U. S. Department of Agriculture

Man Years

Total:	1.8
Professional:	1.2
Other:	.6

Project Description: Part I. In vitro and in vivo studies of the specificity of tuberculins.

Objectives: The overall objectives are concerned with 1) in vivo and in vitro determination of specificity, potency, and identity of tuberculins; 2) identification of organism responsible for human sensitivity to tuberculins; 3) conditions which affect tuberculin hypersensitivity. The project is divided into four parts.

Methods Employed: Part I: Standardization of the skin test reactivity of tuberculins from various species mycobacteria was accomplished in homologously sensitized guinea pigs by determining the dose needed to induce a reaction size of 150-200 mm^2. Reactivities of the tuberculins were compared in

homologous and heterologous systems. In vitro blast cell transformation studies were performed with lymphocytes from lymph nodes of sensitized guinea pigs and from peripheral blood of sensitive humans.

Major Findings: Part I: In sensitized guinea pigs reactivity was always greatest with the homologous tuberculin. Degree of cross reactivity varied greatly with the various tuberculins. The in vitro blast cell transformation procedure correlated well with skin tests both in guinea pigs and in humans.

Project Description: Part II. Mycobacterial Species of Series I-International Working Group on Mycobacterial Taxonomy (IWGMT). Information pertaining to specificity of tuberculins derived from these organisms will be obtained.

Methods Employed: Part II: Organisms of the various species have been grown on Lowenstein-Jensen and oleic-acid albumin media and growth rates and characteristics have been observed. Tuberculins have been prepared from cultures grown in Long's synthetic medium.

Major Findings: Part II: Growth rates and characteristics (pigment, texture, etc.) varied greatly among the species of series I organisms. In Long's medium, several of the organisms produced a high molecular weight substance which could be removed and purified by centrifugation. Thus these organisms can be classified into two groups: those that produce the high molecular weight substance and those which do not.

Project Description: Part III. Tuberculin Hypersensitivity in the Rat, Dog, and Cow.

Methods Employed: Part III: a) Effects of dose and route of administration of BCG in water-in-oil emulsion on the induction of tuberculin hypersensitivity and antibody in the rat have been determined. b) Dogs were treated with phytohemagglutinin (PHA), imuran, and a combination of these drugs and sensitized with bovine serum albumin (BSA) in complete Freund's emulsion. Dogs were skin tested with tuberculin and BSA. Antibody was measured by hemagglutination and precipitation procedures. c) Tuberculin positive cows were bled and their lymphocytes were challenged in vitro with tuberculin. Blast cell formation of lymphocytes as a measure of sensitivity was determined by the incorporation of H^3-labeled thymidine.

Major Findings: Part III: White cells of cows and dogs responded to in vitro challenge with tuberculin by incorporating high levels of H^3-thymidine. Normal sensitized dogs reacted well to skin test challenge with tuberculin. Those dogs treated with both imuran and PHA reacted poorly or not at all. Dogs on only PHA or imuran were not as reactive as normal sensitive dogs but more reactive than dogs on both PHA and imuran. Rats became sensitive to tuberculin and produced good hemagglutinating antibodies regardless of the route. Ten mg BCG was determined to be the optimal sensitizing dose.

Project Description: Part IV. Effects of Frequent Skin Testing on Tuberculin Sensitivity and Antibody Formation.

Methods Employed: Part IV: Guinea pigs were sensitized with BCG and skin-tested weekly with doses of tuberculin ranging from 5 µg to 200 µg. Frequent bleedings were made from the retroorbital plexus for hemagglutination and precipitin tests.

Major Findings: Part IV: Skin sensitivity to tuberculin increased with each test for the first 4 weeks and then fluctuated greatly between high peaks and low valleys with a tendency toward gradual desensitization.

Significance to Bio-Medical Research and the Program of the Division: By performing a sensitivity profile of degree of reactivity of cells of humans or animals to various tuberculins it may be possible to determine the infecting organism responsible for sensitivity. A reliable identification test for tuberculin may be developed *in vivo* in guinea pigs or *in vitro* with their lymphocytes. The *in vitro* test offers an efficient procedure for determining the potency of diagnostic skin test tuberculins.

Proposed Course of Project: Emphasis on the *in vitro* procedure for determining sensitivity has been placed in studies with humans and animals. *In vitro* and *in vivo* procedures for determination of specificity, potency and identity of tuberculins will be extended.

Honors and Awards: None

Publications:

Chaparas, S. D., Baer, H., and Godfrey, H.: *In vivo* and *in vitro* Studies on Tuberculin Active Polysaccharide and Protein Fractions 1968. *Zeitschrift für Immunitatsförschung* (In Press).

Serial No. DBS 102
1. Bacterial Products
2. Allergenic Products
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunology of the Delayed Contact Sensitivity Induced by Catechols Related to and Contained in Poison Ivy Extracts

Previous Serial Number: SAME

Principal Investigators: Harold Baer, Ph. D.
Henry Godfrey, M. D.

Other Investigators: C. J. Maloney, Ph. D. (OD)

Cooperating Units: Radiation Safety Department (CC)
Office of the Director

Man Years

Total:	1.9
Professional:	1.0
Other:	0.9

Project Description:

Objectives: (1) To examine the composition and to establish a method for the standardization of poison ivy extracts and related plants such as poison oak and sumac. (2) To determine whether poison ivy or oak extracts, or the purified active principles, can hyposensitize humans. (3) To determine the relationship of structure of catechols to their effectiveness in inducing delayed contact sensitivity, and to study the mechanism of delayed contact sensitivity.

Methods Employed: (1) Guinea pigs, sensitized to various synthetic catechols, were used to evaluate the relationship of catechol structure to immunologic specificity. (2) Poison ivy extract (obtained commercially) and pure urushiol from poison ivy and oak (from Columbia University) were prepared in olive oil solution. The potency of these solutions was determined by assay in sensitive guinea pigs. Poison ivy materials were sent to Dr. Auerbach and the poison oak to Dr. Epstein for administration to sensitive humans under contracts PH-43-64-885 and PH-43-68-632.

Urushiol, obtained from poison ivy leaves by Professor Dawson, was catalytically reduced in the presence of tritium to yield radioactive pentadecylcatechol (PDC). This material was used to trace the distribution of PDC in guinea pigs after topical application.

Studies were carried out with guinea pigs sensitized to dinitrofluorobenzene (DNFB) and dinitrothiocyanatebenzene (DNTB) as models of delayed sensitivity. Conjugates of DNFB and DNTB (as well as PDC) with bovine, human and guinea pig serum have been prepared in an effort to detect haptene specific antibodies in appropriately sensitized animals.

Major Findings: (1) Specificity of the delayed reaction, which was previously shown to be mainly associated with the hydrocarbon side-chain, has been demonstrated to depend on the size and shape of the side-chain. (2) Parenteral and oral administration of poison ivy extract to sensitive humans has not resulted in a reduction in their sensitivity. Studies with the purified materials have just started and results are not available. (3) Studies with DNFB revealed the effect of ratio of haptene to protein on the ability of conjugates to induce various immunologic responses.

Significance to Bio-Medical Research and the Program of the Division: These studies are providing information on the mechanism of sensitization by catechols and on the relationship of chemical structure to ability to induce delayed contact sensitivity; also, on the relation of the active ingredients of the various poisonous species of the genus Rhus and the extent to which the active ingredients cross-react.

Proposed Course of Project: The project will be continued until all major objectives have been reached.

Honors and Awards: None

Publications:

Baer, H., Watkins, R. C., Kurtz, A. P., Byck, J. S., and Dawson, C. R.: Delayed Contact Sensitivity to Catechols. II Cutaneous Toxicity of Catechols Chemically Related to the Active Principles of Poison Ivy. J. Immunol. 99: 365-369, Aug. 1967.

Baer, H., Watkins, R. C., Kurtz, A. P., Byck, J. S., and Dawson, C. R.: Delayed Contact Sensitivity to Catechols. III The Relationship of Side-Chain Length to Sensitizing Potency of Catechols Chemically Related to the Active Principles of Poison Ivy. J. Immunol. 99: 370-375, Aug. 1967.

Serial No. DBS 119
1. Bacterial Products
2. Allergenic Products
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Standardization of Allergenic Extracts

Previous Serial Number: SAME

Principal Investigators: Harold Baer, Ph. D.
Henry Godfrey, M. D.

Other Investigators: Donald E. Hill (OD)
Philip Norman, M. D., Johns Hopkins
Lawrence Lichtenstein, M. D., Johns Hopkins

Cooperating Units: Office of the Director
Johns Hopkins Medical School

Man Years

Total:	0.7
Professional:	0.5
Other:	0.2

Project Description:

Objectives: To measure the presence, stability, and quantity of Antigen E in commercial ragweed extracts. (2) To immunize rabbits with extracts of various pollens to produce antisera capable of estimating the quantity of extract in mixed commercial extracts. (3) To prepare the lipids of pollen and determine whether delayed sensitivity to these lipids can be induced in guinea pigs.

Methods Employed: (1) Gel diffusion to establish the presence of Antigen E in ragweed extracts and to estimate the quantity present. (2) Skin tests in humans and histamine release studies to determine whether the Antigen E content can be correlated with dermal activity in humans. (3) Ether extraction of pollen to produce lipids, and sensitization of rabbits with the extracts of the lipid free pollen and guinea pigs with the lipid portion.

Major Findings: The quantity of Antigen E in commercial ragweed extracts was determined and in many, but not all, instances there was a correlation between protein nitrogen units (PNU) and Antigen E content.

The skin test activity of commercial extracts was determined at Johns Hopkins by Dr. Philip Norman and there appeared to be two groups of extracts, one with approximately 10 times the dermal activity of the other; both groups had the same nominal PNU value.

Ether extracts of pollen have induced delayed sensitivity in guinea pigs. Whether this is due to lipid or protein is not yet certain.

Significance to Bio-Medical Research and the Program of the Division: If the Antigen E content of ragweed extracts can be related to skin-reactivity and histamine release activity of these extracts it may be indicated to require that commercial ragweed extracts contain a specified amount of Antigen E.

If gel-diffusion can be used to determine the presence of a specific pollen extract, it may be possible to determine whether commercial extracts that are mixtures actually contain the extracts claimed on the label.

Proposed Course of Project: To continue until objectives are attained.

Honors and Awards: None

Publications: None

Serial No. DBS 134
1. Bacterial Products
2. Allergenic Products
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Standardization of Methods of Skin Testing Humans for Allergy

Previous Serial Number: NONE

Principal Investigators: Harold Baer, Ph. D.
William B. Grater, M. D.

Other Investigators: B. A. Berman, M. D.
R. Hale, M. D.
M. Scherr, M. D.
W. Swinney, M. D.
C. J. Maloney, Ph. D. (OD)

Cooperating Units: Office of the Director
Committee on Standardization of the American College of Allergy

Man Years

Total:	0.2
Professional:	0.2
Other	0.0

Project Description:

Objectives: It was the purpose of the Committee to attempt to determine whether stringently controlled conditions could improve quantitation of skin-testing of allergic patients.

Methods Employed: Microliter syringes were substituted for regular syringes in order to deliver accurately 0.01 ml intradermally.

The skin of various individuals may respond differently, therefore histamine response of various patients was determined. When the allergenic extract was used for testing, histamine was administered simultaneously as a control.

Major Findings: The data obtained by the various physicians were analyzed at DBS. In the first stage histamine response alone was measured. The analysis revealed that, statistically, all individuals showed a similar dose response curve to histamine with the same slope. The reactivity was independent of physician, geographical location, age of patient, or whether the patient was or was not allergic.

Significance to Bio-Medical Research and the Program of the Division: At present, many allergists do not consider skin testing to be sufficiently quantitative and therefore useful only for a rough evaluation of sensitivity. If skin testing could be demonstrated to be quantitative it could have value for estimating potency of extracts and for directly comparing the reactivity of various extracts. It might be useful for determining the efficacy of therapy and for other quantitative studies in the allergic response.

Proposed Course of Project: To continue to collect data to determine the degree of precision and reproducibility that can be attained in skin testing.

Honors and Awards: None

Publications: None

Serial No. DBS 103
1. Bacterial Products
2. Allergenic Products
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mycotic and Parasitic Diagnostic Skin Test Reagents

Previous Serial Number: SAME

Principal Investigators: Sotiros D. Chaparas, Ph. D.
Milford Lunde, M. S. (NIAID)

Other Investigators: None

Cooperating Units: Laboratory Parasitology, NIAID

Man Years

Total:	.3
Professional:	.3
Other:	.0

Project Description:

Objectives: To develop suitable laboratory procedures for use in potency testing of diagnostic skin test reagents.

Methods Employed: 1. Guinea pigs are sensitized with extracts of axenically grown Entamoeba histolyticum (histolyticin) incorporated in complete Freund's adjuvant. Skin testing with histolyticin is done six weeks later. Antibody is detected by hemagglutination and by precipitation in gel.

2. White cells obtained from peripheral blood of histoplasmin sensitive humans are cultured in the presence of antigen and later pulsed with tritiated thymidine. Lymphocyte transformation by antigen is noted.

Major Findings: 1. Entamoeba histolyticum extract (histolyticin) induced good sensitization and antibody formation in guinea pigs.

2. Lymphocytes from humans skin test reactive to histoplasmin were transformed by exposure to this antigen. Lymphocytes from non-reactive humans did not respond.

Significance to Bio-Medical Research and the Program of the Division:
1. Potency of histolyticin can be assayed in sensitive guinea pigs.
2. Sensitivity of human beings can be demonstrated by *in vitro* procedures, thus skin testing in humans can be avoided.

Proposed Course of Project: Potency test procedures for other diagnostic skin test antigens shall be investigated by the guinea pig system and by the *in vitro* lymphocyte transformation procedure.

Honors and Awards: None

Publications: None

Serial No. DBS 120
1. Bacterial Products
2. Allergenic Products
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Pathogenesis of Lesions Induced by Antigens in Water-in-Oil Emulsions

Previous Serial Number: SAME

Principal Investigator: Sotiros D. Chaparas, Ph. D.

Other Investigators: Robert M. Friedman, M. D. (NCI)
Ruth L. Kirschstein, M. D. (LP)

Cooperating Units: Laboratory of Pathology
National Cancer Institute

Man Years

Total:	.2
Professional:	.2
Other:	.0

Project Description:

Objectives: To investigate the role of age on the development of lesions in guinea pigs.

Methods Employed: Guinea pigs of various ages were sensitized subcutaneously or intradermally in the nuchal area with heat killed BCG organisms incorporated in either saline or water-in-oil emulsion.

Major Findings: The age of the guinea pig was found to be important in the ability to develop delayed skin reactivity and subcutaneous nodules in response to sensitizing doses of BCG organisms in water-in-oil emulsions. Guinea pigs sensitized at birth were unable to display a tuberculin skin reaction until they became adult animals. This unreactivity did not appear to be due to any difference in the rate of elimination of I^{125} labeled tuberculin from the skin of very young animals.

Significance to Bio-Medical Research and the Program of the Division: These studies lend some insight as to the nature of lesions that may arise when killed organisms such as BCG are incorporated in oil adjuvants.

Proposed Course of Project: This experiment is now terminated. The results will be analyzed and written up for publication.

Honors and Awards: None

Publications: None

Serial No. DBS 101
1. Bacterial Products
2. Allergenic Products
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Tuberculosis Vaccine

Previous Serial Number: SAME

Principal Investigator: Sotiros D. Chaparas, Ph. D.

Other Investigator: Mrs. Sally Hedrick

Cooperating Units: None

Man Years

Total:	0.2
Professional:	0.1
Other:	0.1

Project Description: The status of this project is essentially in the state it was last year. BCG vaccines submitted for release are counted for viability.

Serial No. DBS 12
1. Bacterial Products
2. Bacterial Toxins
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigation of Tetanus Toxoids for Prevention of Neonatal Tetanus

Previous Serial Number: SAME

Principal Investigators: Margaret Pittman, Ph. D.
Michael F. Barile, Ph. D.
M. Carolyn Hardegree, M.D.

Other Investigators: Frank Schofield, M.D., New Guinea

Cooperating Unit: Department of Public Health, Territory of Papua and New Guinea

Man Years

Total:	0.6
Professional:	0.3
Other:	0.3

Project Description:

Objectives: To determine the persistence of antitoxin and the booster response in New Guinea women immunized since 1962 with adjuvant and plain tetanus toxoids.

Methods Employed: The mouse neutralization test was used to determine antitoxin levels of the sera of the women.

Major Findings: The mean antitoxin titer of sera from patients boosted with $AlPO_4$ adsorbed toxoid (57 patients) was 2.29 u/ml while the mean antitoxin titer of sera from patients boosted with plain toxoid was 1.55 u/ml (57 patients).

Significance to Bio-Medical Research and the Program of the Division: The field results provide information on the relation of human antitoxin response to the laboratory assayed potency of tetanus toxoids. The findings are applicable to the potency and safety specifications for tetanus toxoid.

Proposed Course of Project: (1) To publish the laboratory and clinical findings. (2) To complete studies to determine duration of the protective antitoxin level and booster response to adsorbed and plain toxoids. (3) To continue studies to determine cause of untoward reactivity of oil adjuvants (Proj. No. 15).

Honors and Awards: None

Publications: None

Serial No. DBS 13
1. Bacterial Products
2. Bacterial Toxins
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A Study of the Antigenic Substances of *Clostridium tetani*

Previous Serial Number: SAME

Principal Investigator: M. Carolyn Hardegree, M. D.

Other Investigators: A. E. Palmer, D.V.M. (LP)
J. J. Helprin, Ph. D. (LBB)

Cooperating Units: Laboratory of Pathology
Laboratory of Biophysics and Biochemistry

Man Years

Total:	1.0
Professional:	0.4
Other:	0.6

Project Description:

Objectives: (1) To determine and separate the antigens present in culture filtrates of *Cl. tetani*. (2) To characterize the *in vivo* and *in vitro* activities of these substances. (3) To determine their relationship to optimal anti-tetanus immunization.

Methods Employed: Column chromatography, zone electrophoresis, gel diffusion, immunoelectrophoresis, enzyme assays, plasma hemoglobin determination, thin layer chromatography, gas chromatography, animal bioassay, animal immunization.

Major Findings: (1) Esterase activity detected by the use of Arlacel A, olive oil and peanut oil was enhanced by the addition of human 7S gamma globulin. This does not appear to be an immune phenomenon. (2) Preliminary data suggest the hemolysin may be cardiotoxic for monkeys.

Significance to Bio-Medical Research and the Program of the Division: It is expected that the investigation will contribute to an understanding of the role of antibodies against *Cl. tetani* antigens other than the neurotoxin. These antibodies may be necessary for the prophylaxis and the treatment of tetanus. Findings will provide fundamental information for the control of these products.

Proposed Course of Project: Studies will be continued on (1) the isolation and recovery of components of the culture filtrates, (2) the continued characterization of the *in vivo* and *in vitro* effects of the hemolysin, (3) the relation of the esterase to adverse reactions of antigens in oil adjuvants and the nature of the effect of globulin on the esterase.

Honors and Awards: None

Publications: None

Serial No. DBS 14
1. Bacterial Products
2. Bacterial Toxins
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Standardization of Reference Preparations for Tetanus Toxoids

Previous Serial Number: SAME

Principal Investigators: Margaret Pittman, Ph. D.
Michael F. Barile, Ph. D.
M. Carolyn Hardegree, M. D.
Robert W. Kolb, M.S. (LCA)
Edward B. Seligmann Jr., Ph. D. (LCA)

Other Investigators: None

Cooperating Unit: Laboratory of Control Activities

Man Years

Total:	0
Professional:	0
Other:	0

Project Description:

Objectives: (1) To establish a unitage for a U. S. reference toxoid. (2) To correlate laboratory assayed potency with the antitoxin response of women in New Guinea. (3) To relate these findings to the U. S. potency requirement for tetanus toxoid.

Methods Employed: Mice and guinea pigs were vaccinated with graded doses of tetanus toxoids and their responses were determined (1) by challenge with tetanus toxin and (2) by titration of antitoxin levels of guinea pig sera by the mouse neutralization test.

Major Findings: Findings are being prepared for publication.

Significance to Bio-Medical Research and the Program of the Division: The international reference tetanus toxoid adsorbed provides a basis for comparison of assayed potency of adsorbed tetanus toxoids prepared in all nations. (An international reference for the plain toxoids was designed earlier.) It will be possible to assay the unitage of U. S. tetanus toxoid by use of the correlated U. S. references and to relate laboratory assayed

unitage to antitoxin response of New Guinea women.

Proposed Course of Project: The findings will be prepared for publication. Attempts will be made to determine the feasibility of applying the information to the U. S. requirements for potency of tetanus toxoids.

Honors and Awards: None

Publications:

Pittman, M., Barile, M. F., and Hardegree, M. C.: Comparison of Human Response with Laboratory Assays of Tetanus Toxoid with and without Adjuvants, International Symposium on Adjuvants of Immunity, Utrecht, 1966. In Symposium Series Immunobiological Standardization, vol. 6, Basel/New York, Karger, 1967, pp. 295-302. (Reported as "In Press" in 1966.)

Serial No. DBS 15
1. Bacterial Products
2. Bacterial Toxins
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigation of Toxicity of Oil Emulsified Antigens

Previous Serial Number: SAME

Principal Investigators: M. Carolyn Hardegree, M. D.
Michael F. Barile, Ph. D.

Other Investigators: Ruth Kirschstein, M. D. (LP)
Ronald Clark, Ph. D. (LP)

Cooperating Unit: Laboratory of Pathology

Man Years

Total:	1.6
Professional:	0.85
Other:	0.75

Project Description: Part I. The toxicity of oil adjuvant components and free fatty acids in animals.

Objectives: To determine factors responsible for sterile abscesses in recipients of oil emulsified biologics.

Methods Employed: (1) Free fatty acids of various oil adjuvant components were determined. (2) Mice, rabbits and guinea pigs were injected intraperitoneally, intramuscularly or subcutaneously with components of oil adjuvants and with free fatty acids. The gross and microscopic pathology was observed and the weight change of mice was determined.

Major Findings: (1) Free fatty acids (FFA) in quantities detected in emulsified antigens associated with sterile abscesses did not prevent weight gain in mice or induce granulomas in rabbits. (2) Arlacel A containing comparable levels of FFA did induce granulomas in rabbits. (3) These data suggest that FFA per se are not responsible for sterile abscesses.

Project Description: Part II. The toxicity of oil adjuvant components in cell culture.

Objectives: To examine the use of cell cultures for the evaluation of oil adjuvant component toxicity and to evaluate the mechanism of this toxicity.

Methods Employed: (1) Cultures of 6 cell lines were inoculated with various oils and emulsifiers and the cell toxicity was measured. (2) The effect of various diluents on the toxicity of the oils and emulsifiers was determined. (3) A comparison was made between the in vitro and in vivo toxicity.

Major Findings: (1) Preliminary findings indicate that cell cultures can be used to evaluate the toxicity of oil adjuvant components, especially the emulsifier, Arlacel A. Cell culture toxicity of several lots of Arlacel A correlated well with the toxicity as determined by the in vivo intraperitoneal test. (2) Certain diluents enhance the toxicity of Arlacel A for cell culture and for mice. (3) Similar cytopathic effects to Arlacel A were produced in each of the 6 cell lines tested.

Significance to Bio-Medical Research and the Program of the Division: These studies provide information on the reactivity of components of oil emulsified antigens and may be useful in developing specifications for oil emulsified products should the use of oil adjuvants be approved.

Proposed Course of Project: To continue to evaluate various in vivo procedures for the evaluation of the toxicity of the components of oil adjuvants and to continue to evaluate the relationship of the FFA in the emulsifier, Arlacel A, to reactivity. To use cell cultures to evaluate the toxicity of other adjuvant components. To prepare findings for publication.

Honors and Awards: None

Publications:

Hardegree, M. Carolyn and Kirschstein, Ruth: The Toxicity of Free Fatty Acids and Arlacel A. Presented at VI International Congress of Allergology, Session on Repository Therapy, Pro and Con, Montreal, Canada. Annals of Allergy, in press.

Serial No. DBS 16
1. Bacterial Products
2. Bacterial Toxins
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Role of Transitional L-Forms of Bacteria in Human Disease

Previous Serial Number: SAME

Principal Investigators: Michael F. Barile, Ph. D.
Thomas C. Francis, D.D.S. (NIDR)
Edward A. Graykowski, D.D.S., M. D. (NIDR)

Other Investigators: Joost J. Oppenheim, M. D. (NIDR)
Constance DeYoung, M.S. (NIDR)

Cooperating Unit: National Institute of Dental Research

Man Years

Total:	0.1
Professional:	0.1
Other:	0.0

Project Description:

Objectives: To identify and characterize the transitional L-forms of streptococci isolated from aphthous lesions and to examine their role in the pathogenesis of recurrent aphthae.

Methods Employed: Human and guinea pig lymphocyte cultures were inoculated with various antigens from pathogenic and non-pathogenic streptococci and incorporation of tritiated thymidine was determined.

Major Findings: (1) The mitotic response of human lymphocyte cultures was greater to beta-hemolytic pathogenic streptococci than to alpha-hemolytic non-pathogenic streptococci. (2) Cell wall extracts of streptococci containing group specific carbohydrates and purified type M protein were better stimulants than were the intact streptococcal cells. (3) Pathogenic Group A streptococci did not stimulate cultures of lymphocytes from normal guinea pigs but did stimulate cultures of lymphocytes from immunized guinea pigs.

Significance to Bio-Medical Research and the Program of the Division: These studies will be useful in establishing the etiology of recurrent aphthae and, subsequently, may provide a means by which this disease may be arrested perhaps by vaccination. These studies are related to prevention and detection of Mycoplasma in biologics and provide suitable materials to test new and improved methods for primary isolation and detection of Mycoplasma and L-forms of bacteria.

Proposed Course of Project: To pursue studies to improve methods for isolation and detection of L-forms of bacteria in biologics.

Honors and Awards: None

Publications:

Barile, M. F., Francis, T. C., and Graykowski, E. A.: Streptococcus sanguis in the Pathogenesis of Recurrent Aphthous Stomatitis. In Guze, L., (Ed.): Protoplasts, Spheroplasts and L-forms, Baltimore, Md., Williams & Wilkins Co., 1968, pp. 444-456. (Reported as "In Press" in 1967.)

Francis, T. C., Oppenheim, J. J., and Barile, M. F.: Lymphocyte Transformation of Streptococcal Antigens in Guinea Pig and Man. In Ricke, W. O. (Ed.): Proceedings of the Third Annual Lymphocyte Culture Conference, New York, New York, Appleton-Century-Crofts, Inc. (In Press.)

Serial No. DBS 17
1. Bacterial Products
2. Bacterial Toxins
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mycoplasma in Human Disease and Biologics

Previous Serial Number: SAME

Principal Investigator: Michael F. Barile, Ph. D.

Other Investigators: Brigid G. Leventhal, M. D. (NCI)
Donald B. Riggs
Marion W. Grabowski

Cooperating Unit: Medicine Branch, NCI

Man Years

Total:	1.8
Professional:	0.45
Other:	1.35

Project Description:

Objectives: (1) To examine the biology, metabolism, and pathogenicity of Mycoplasma. (2) To develop new methods or improve existing methods for primary isolation and detection of Mycoplasma as they pertain to the test for Mycoplasma required for certain biologic products. (3) To investigate the role of Mycoplasma in diseases of man and animals.

Methods Employed: Human lymphocyte cultures were inoculated with phytohemagglutinin (PHA) and PHA in combination with cultures of 6 Mycoplasma species, respectively, and the effects on the incorporation of tritiated thymidine were determined.

Major Findings: (1) PHA-induced transformation of human lymphocyte cultures was inhibited by arginine-using Mycoplasma but not by dextrose-using Mycoplasma. (2) Inhibition was prevented with addition of arginine, or with complete medium but not by arginine-free medium. (3) Arginine depletion by certain Mycoplasma deprives lymphocyte cultures of this essential amino acid resulting in impaired lymphocyte function.

Significance to Bio-Medical Research and the Program of the Division: These studies may provide information on the pathogenicity of mycoplasmas for man. The improved culture procedures developed for the isolation of mycoplasma from human specimens are applicable to the isolation and detection of mycoplasmas in biologics which are subject to mycoplasma-contamination risk.

Proposed Course of Project: To pursue studies to improve methods for primary isolation of Mycoplasma from biologics and from tissues associated with disease. To consider assay methods for requirements for Mycoplasma vaccine products.

Honors and Awards: None

Publications:

Barile, M. F.: Mycoplasma and Leukemia. Ann. N. Y. Acad. Sci. 144: 557-572, July 1967. (Reported as "In Press" in 1967.)

Barile, M. F.: Mycoplasma and Cell Cultures. In Merchant, D. (Ed.): Cell Culture for Virus Vaccine Production, J. Nat. Cancer Inst. Monograph No. 29 (In Press).

Serial No. DBS 22
1. Bacterial Products
2. Bacterial Vaccines
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Cholera and Cholera Vaccine

Previous Serial No.: SAME

Principal Investigator: John C. Feeley, Ph. D.

Other Investigators: Margaret Pittman, Ph. D.
Carl E. Miller, D.V.M.
Charles O. Roberts
Thomas F. Brown

Cooperating Units: NIH Cholera Advisory Committee
U. S. Japan Cooperative Medical Science Program
Pakistan-SEATO Cholera Research Laboratory
(NIH Research Agreement 196802)
National Communicable Disease Center
Johns Hopkins University School of Medicine
University of Maryland School of Medicine

Man Years

Total:	2.3
Professional:	1.6
Other:	0.7

Project Description:

Objectives: (1) Establishment of a meaningful standard of potency and definition of other requirements pertinent to standardization of cholera vaccine; (2) Evaluation of the possible significance of cholera toxin antigen as an immunizing agent against this disease; (3) Determination of the relative roles of antibacterial and antitoxic antibodies in passive protection in the infant rabbit model; (4) Characterization of subspecies characteristics of Vibrio cholerae strains from recent outbreaks of cholera.

Methods Employed: Quantitative potency assays of vaccines were carried out in mice. Guinea pigs and rabbits were immunized with experimental toxins and toxoids to assess the formation of antitoxic and antibacterial antibodies as measured primarily by agglutination and vibriocidal tests and the skin

permeability factor (PF) neutralization technique. Human sera from volunteers receiving a U. S. licensed cholera vaccine containing a small amount of PF toxin were similarly assayed. Routine bacteriological and serological methods were employed in examination of cultures.

Major Findings: Subproject 1: Standardization of cholera vaccine. This laboratory continues to collaborate with the Pakistan-SEATO Cholera Research Laboratory (PSCRL) in the selection and assay of vaccines employed in controlled field trials. Results of field trials to date suggest that an effect of vaccine potency in this endemic area is detectable only in the age group of 5 years or less. When compared on the basis of the single human dose employed, the vaccine used in the 1963-64 field trials was approximately twice as potent against Ogawa challenge and six times as potent against Inaba challenge than the vaccine employed in the 1966 trial. While differences between these vaccines in potency against Ogawa challenge were not striking, the considerable differences in potency against Inaba challenge were reflected in the results of the field trial in children under five years of age. A single dose of the higher potency vaccine gave 75-80% protection in the 1963 and 1964 trials, while a single dose of the lower potency vaccine gave only 24% protection in 1966. Nearly all cases were due to Inaba serotype, hence no evaluation of the relationship of potency and efficacy against Ogawa serotype is possible for these vaccines.

At this time assays for potency and freedom-from-toxicity are in progress on freeze-dried monovalent Ogawa and Inaba cholera vaccines procured under contract and scheduled to be employed in field trials at the PSCRL in Fall 1968.

Subproject 2: Cholera Toxin Studies. Toxoids prepared by mild heating and formalin treatment have been assayed along with the parent toxin for antigenicity in guinea pigs and rabbits. Heat-inactivated toxoid was less antigenic than either toxin or formalinized toxoid. The latter toxoid was surprisingly found to be 3 to 5 times more antigenic than the parent toxin in both guinea pigs and rabbits. A single dose of toxin or toxoid was found to induce a prolonged slowly rising antitoxin response in guinea pigs which did not reach a peak until 120 days after initial stimulus. Similar studies are in progress in rabbits.

In collaboration with Dr. C.C.J. Carpenter and associates at Johns Hopkins School of Medicine, it has been shown that immunization of dogs with the above formalinized-toxoid significantly inhibits accumulation of fluid in the bowel following intraluminal challenge with cholera toxin. Studies on protective effect against live vibrio challenge are in progress.

In a cooperative study with Dr. Eugene Gangarosa of NCDC, antitoxin titrations were performed on sera obtained from human volunteers immunized with a routine U. S. licensed cholera vaccine which contains a small amount (428 Bluing Doses/ml) of PF toxin. It was determined that 13 of 22 subjects showed a slight but detectable antibody response 28 days after immunization. Later

bleedings and the effect of booster doses could not be evaluated. Additional sera from subjects vaccinated with this vaccine and other non-toxin containing vaccines will be provided to us by Dr. Richard Hornick of the University of Maryland.

A publication based on the use of cholera toxin prepared in this laboratory is listed below.

Subproject 3: Passive protection studies in the infant rabbit. While little progress was made in this area during this reporting period, experiments are contemplated to assess the relative roles of antibacterial and antitoxic antibodies in passive protection of the infant rabbit against disease due to V. cholerae infection. Experience to date suggests that only sera with antitoxic activity are protective in this test system, but such sera contain antibacterial antibodies as well.

Attempts to detect either vibriocidal or antitoxic antibodies in intestinal secretions of infant rabbits passively immunized with sera having high titers of these antibodies were unsuccessful.

Subproject 4: Characterization of strains of Vibrio cholerae. Twelve strains isolated in Vietnam in 1967 were examined. Eight were of serotype Inaba and 4 were Ogawa. All belonged to type 4 in our previously described scheme. These strains were of special interest because 3 were reported to belong to type 1 (e.g. "classical" cholera vibrios instead of "El Tor" vibrios); however this was not confirmed.

Significance to Bio-Medical Research and the Program of the Division: Continued efforts are necessary to establish a correlation of laboratory potency and efficacy in man. Data available are suggestive that a correlation may exist at least for the Inaba serotype, while inadequate information from the field is available for the Ogawa serotype. A conclusive demonstration would require a field trial in which vaccines of differing potency are employed simultaneously. The projected field trials employing monovalent vaccines may yield important information on cross-protection in man.

It is becoming more and more evident that presently available vaccines cannot be expected to yield a high degree of long-term protection, at least in the very young age group in which the incidence of clinical cholera is highest in endemic situations such as East Pakistan.

The possible significance of antitoxic immunity in human cholera remains undetermined. Vaccines tested in the field thus far do not stimulate antitoxin and are presumed to protect by an antibacterial mechanism. Development of suitable toxoid immunizing agents for human use are needed to test this hypothesis. The studies reported suggest that toxoid may be more antigenic than toxin, and this may be of significance in view of some suggestions that toxoiding is unnecessary due to the apparent inocuity of cholera toxin given by the parenteral route. Also, it remains to be determined whether the permeability factor toxin is actually the sole moiety responsible for the disease.

Several observations are suggestive that other factors are involved.

It is felt that continued monitoring of the characteristics of representative Vibrio cholerae strains from different epidemics is worthwhile in order to evaluate any possible changes which may be proposed in composition of the vaccine. While numerous suggestions for inclusion of El Tor strains in the vaccine have been made, the facts do not appear to necessitate this action.

Proposed Course of the Project: Studies will be continued along the lines outlined above.

Honors and Awards: Dr. Feeley has been appointed to the World Health Organization Expert Panel on Bacterial Diseases.

Publications:

Carpenter, C.C.J., Sack, R. B., Feeley, J. C., and Steenberg, R. W.: Site and characteristics of electrolyte loss and effect of intraluminal glucose on experimental canine cholera. J. Clin. Invest. (In press).

Serial No. DBS 104
1. Bacterial Products
2. Bacterial Vaccines
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Pertussis Vaccine

Previous Serial Number: SAME

Principal Investigators: Margaret Pittman, Ph. D.
Charles R. Manclark, Ph. D.
Ronald G. Clark, Ph. D. (LP)

Other Investigators: Ruth L. Kirschstein, M. D. (LP)
Duane B. Gainsburg, M. D. (NINDB)
James F. Marshall

Cooperating Units: Laboratory of Pathology
Surgical Pathology Branch (NINDB)
Pharmaceutical Manufacturers Association

Man Years

Total:	2.6
Professional:	1.0
Other:	1.6

Project Description:

Objectives: The overall objective is that the pertussis vaccine component of products issued by U. S. licensed establishments has optimum properties: adequate and stable potency through the dating period and relative freedom from untoward reactivity. Objectives of current studies are (1) to relate laboratory assayed reactivity of vaccines and factions of the constituent bacteria, as determined by multiple tests, to clinical reactivity, and (2) to find the cause of the neurological reactions that on rare occasions follow pertussis vaccination and to develop a laboratory assay that measures the reactive factor should it be identified.

Methods Employed: Mouse potency tests to determine adequacy and stability of potency. Analysis of results at intervals to determine any reflected trend. Measurement of pertussis vaccine toxicity (reactivity) by determination of the weight changes in mice, the lethal dose in mice, histamine sensitizing activity, necrotizing activity in the skin of rabbits and suckling mice, capacity to induce edema in foot pad of the rat, pyrogenicity, etc. Induction of allergic encephalomyelitis or encephalopathy in monkeys, rats, mice and

guinea pigs. Electroencephalograms and blood and spinal fluid chemistry of monkeys and histopathology of all animals.

The laboratory assayed reactivities of two well characterized pertussis vaccines are currently being determined in cooperation with manufacturers of pertussis vaccine and will be related to reactivity in children. The clinical study is under the supervision of Dr. Harry M. Meyer (LVI) and a representative of the PMA.

Major Findings: Subproject 1: No significant changes in potency and toxicity of pertussis vaccines submitted for release were noted. The histamine sensitizing factor (HSF) unitage of the U. S. Standard Pertussis Vaccine Lot 6 was assayed in terms of the unit as defined by the Netherlands.

Subproject 2: The collaborative laboratory assays of the study vaccines have not been completed by all participants.

Subproject 3: Although allergic encephalopathy has been induced in monkeys and rats, the experiments started a few months ago are still exploratory in nature. Hence results can not yet be related to the objective of the study.

Significance to Bio-Medical Research and the Program of the Division: The study is related directly to the control of the potency and safety of pertussis vaccine and the relation of laboratory assayed toxicity to untoward reactivity in children. The establishment of an HSF unit will permit evaluation of the HSF content to reactivity of the vaccine. Encephalopathy that follows vaccination in rare instances is a distressing reaction and the experimental studies are directed towards the elucidation of the cause. This project also provides basic immunological information on Bordetella pertussis.

Proposed Course of Project: To continue the project as outlined above.

Honors and Awards: 1. Lecture: "Pertussis Vaccine" presented by M. Pittman at Howard University College of Medicine, January 25, 1968.

2. Paper by Gardner, R., Bohner, H., and Pittman, M. entitled, "Reactivity of germ-free and conventional mice to pertussis vaccine", presented at X International Congress of the Permanent Section of Microbiological Standardization, Prague, September 19-23, 1967.

Publication:

Pittman, M.: Mouse Strain Variation in Response to Pertussis Vaccine and Tetanus Toxoid, International Symposium on Laboratory Animals, London, 1966. In Symposium Series Immunobiological Standardization, vol. 5, Basel/New York, Karger, 1967, pp. 161-166.

Serial No. DBS 116
1. Bacterial Products
2. Bacterial Vaccines
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Typhoid Vaccine

Previous Serial Number: SAME

Principal Investigator: Vito M. Esposito, Ph. D.

Other Investigators: John C. Feeley, Ph. D.
Margaret Pittman, Ph. D.

Cooperating Units: None

Man Years

Total:	2.0
Professional:	1.0
Other:	1.0

Project Description:

Objectives: The principal objective is to develop a potency assay that reflects efficacy of all types of typhoid vaccines for man. The current objectives are to determine the mechanism(s) of protection in the mouse evoked by immunization with typhoid vaccines and the role of the constituent antigens in these processes.

Methods Employed: Routine serological and bacteriological procedures, mouse protection tests and various physico-chemical techniques are being employed.

Major Findings: Based on earlier work reported in the literature an assay for quantitating the Vi antigen content of typhoid vaccine has been devised. The sensitivity of this turbidimetric test imposes certain problems which may limit its use for control activities. These limitations do not affect use of the assay for qualitative purposes, but only the quantitative aspects. Evaluation of other possible assay systems has been hampered by such problems as anticomplementary and nonprecipitating sera.

Three strains of mice injected with typhoid vaccine showed significant differences in formation of Vi antibody but not in protective response against

challenge with Salmonella typhosa. When injected with soluble Vi antigen the three strains differed significantly in response as measured both by the in vitro and in vivo tests. The immunogenic potential of Vi antigen in the New Zealand white rabbit was significantly influenced by the physical state in which it was presented to the animal. These results emphasized that three factors are involved in the antibody response to Vi antigen, (i) the host, not only the species but the strain, (ii) nature of the antigen, and (iii) route of immunization.

Active cutaneous anaphylaxis was shown to be an in vivo method of demonstrating antibody response of mice vaccinated with typhoid vaccine or Vi antigen and provides a means of detecting antibody formation when levels are too low for in vitro assays. The NIH strain of mouse reacted equally well to typhoid vaccine 6A and Vi antigen while the CFW strain did not respond to either antigen.

Passive cutaneous anaphylaxis (PCA) was demonstrated to be an effective method of demonstrating an antibody response of rabbits to Vi antigen and typhoid vaccine. Mouse antisera did not invoke PCA reactions in guinea pigs; however, they did appear to produce an Arthus type reaction. The ability of rabbit anti-Vi sera to induce PCA appears to be dependent on the physical state (i.e. soluble or erythrocyte absorbed) of Vi antigen used to stimulate antibody formation.

A method for purification of Vi antigen has been developed using ion exchange resins. This method offers the advantage of eliminating drastic treatments (acid hydrolysis, etc.) which have been used by others. The antigenicity of this Vi preparation in rabbits was comparable to other preparations but induced a greater level of protection in mice challenged with S. typhosa.

Preliminary evidence indicates the production of more than one form of globulin in response to immunization with Vi antigen. 7S and 19S antibodies were produced simultaneously in both mice and rabbits.

Significance to Bio-Medical Research and the Program of the Division: In earlier studies it was shown that the U. S. prescribed potency assay reflected human efficacy for acetone and heat-phenolized typhoid vaccines but not for alcoholized vaccines which are high in Vi content. Determination of the influence of the various antigens, particularly Vi antigen, and why the route of vaccination significantly affects the immunological response of mice are indicated in order to develop the test method(s) that reflect human efficacy.

Proposed Course of Project: To continue evaluation of various types of typhoid vaccines with the prime objective to develop a potency assay that reflects the efficacy of all typhoid vaccines for man. In addition, studies are planned to investigate the processes of resistance (immunity) and disease (infection).

Honors and Awards: None

Publications: None

Serial No. DBS 136
1. Bacterial Products
2. Bacterial Vaccines
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Evaluation of Microbially Controlled Animals in Control Testing and Infection

Previous Serial Number: NONE

Principal Investigators: Carl E. Miller, D.V.M.
John C. Feeley, Ph. D.

Other Investigator: Thomas F. Brown

Cooperating Unit: None

Man Years

Total:	1.1
Professional:	0.6
Other:	0.5

Project Description: Part I. Microbially Controlled Animals in Control Testing.

Objectives: To determine the effects of utilization of microbially controlled (i.e. so-called "caesarean-derived barrier sustained" or "specific pathogen-free") animals having a greatly reduced variety of microbial flora on control tests of bacterial vaccines.

Methods Employed: Simultaneous tests for potency and freedom-from-toxicity with various vaccines (e.g. pertussis, typhoid, cholera) are being performed in parallel using "conventional" and microbially controlled mice of the same strain.

Major Findings: Only a small number of comparative tests have been carried out to date due to shortage of required numbers of microbially controlled mice. In comparative tests conducted thus far, microbially controlled mice of the NIH strain have responded similar to conventional mice of this strain in protective response to immunization with pertussis vaccine and to the lethal effect of Salmonella typhosa infection. However, microbially controlled mice were approximately 5 times more resistant than conventional mice to the toxic effects of pertussis vaccine in the freedom-from-toxicity test prescribed for this product. In this respect, they resembled closely the previously reported response of germ-free mice.

No significant difference in the vibriocidal antibody response of these mice was noted following immunization with cholera vaccine.

Project Description: Part II. Role of the Indigenous Microflora in Resistance of Mice to Infection with Vibrio cholerae.

Objectives: To determine which organism or organisms are responsible for natural resistance to gastrointestinal infection with Vibrio cholerae in mice.

Methods Employed: Germ-free mice in isolators are being used to maintain a controlled microbial environment. Pure cultures of various intestinal organisms are being added to determine which are effective in preventing establishment of V. cholerae and which are effective in eliminating V. cholerae from the GI tract of mice.

Mice treated with oral streptomycin to reduce bacterial flora were infected with streptomycin-resistant strains of V. cholerae, Ogawa and Inaba. Cultures were carried out at intervals and typed serologically to determine whether serotype changes previously noted in germ-free mice occur also in this system.

Major Findings: Vibrio cholerae is readily established in the GI tract of germ-free mice without producing illness. It annot be established in the GI tract of conventional mice or microbially controlled mice with greatly reduced variety of microflora consisting mainly of lactobacilli, bacteroides and enterococci. Experiments are currently in progress to assess the role of the latter organisms in natural resistance to infection.

The eventual appearance of rough strains of streptomycin resistant cholera vibrios in chronically infected streptomycin treated mice is much the same as in germ-free mice. However, the conversion of Ogawa serotype to Inaba and Inaba serotype to Ogawa before the appearance of roughness was of much lower frequency than in the germ-free animal.

Significance to Bio-Medical Research and Program of the Division: In view of the present trend toward greater use of microbially controlled animals from commercial and other breeding colonies, the comparative response of these animals must be determined to evaluate their suitability for control testing of biologic products. The importance of this comparison is evident thus far from the observation that a pertussis vaccine could be considered non-toxic if tested in quality controlled animals and still have significant toxicity based on tests with conventional animals.

The selective forces operating in allowing establishment of clinical cholera in some persons and not others have never been fully understood. There is a likelihood that the normal intestinal microflora is at least as important as immunologic mechanisms in natural resistance to this infection. A clearer understanding of the influence of the intestinal microflora in the

establishment of Vibrio cholerae in rodents may suggest some basis for understanding of similar factors in the natural human host.

Proposed Course of Project: Continuation along the lines outlined above.

Honors and Awards: None

Publications: None

Serial No. DBS 138
1. Bacterial Products
2. Bacterial Vaccines
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Selective Breeding to Establish a "Standard" Mouse

Previous Serial Number: NONE

Principal Investigators: Margaret Pittman, Ph. D.
Carl T. Hansen, Ph. D. (DRS)
Charles R. Manclark, Ph. D.

Other Investigators: James F. Marshall
William J. Hinkle (DRS)

Cooperating Unit: Division of Research Services

Man Years

Total:	0.6
Professional:	0.2
Other:	0.4

Project Description:

Objective: To establish a mouse strain that has superior and consistent characters for the potency and toxicity assays of biologics, particularly bacterial vaccines.

Methods Employed: Forty or more pairs of mice have been selected. The progeny of each is being tested for ability to be sensitized to histamine by pertussis vaccine. Mice with the highest ability are being selected for further breeding. It is anticipated that it will take two to three years to establish the colony.

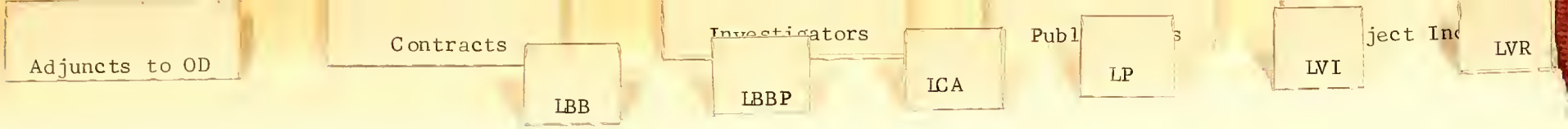
Major Findings: 1. Mice can be selected and bred for an inherited sensitivity to histamine following injection with pertussis vaccine. 2. Mice in the second generation of selective breeding have demonstrated a significant increase in sensitivity to histamine following injection with pertussis vaccine.

Significance to Bio-Medical Research and the Program of the Division: The availability of a "standard" mouse for potency assays of vaccines would decrease variability in tests and give greater assurance of the validity of the estimate of the potency of a biologic product.

Proposed Course of Project: (1) To continue the study as described above. (2) To determine the nature of the genetic markers for histamine sensitivity and resistance and their inheritance in NIH BxS mice. (Hansen)

Honors and Awards: None

Publications: None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Annual Report of the
Laboratory of Biophysics and Biochemistry
Division of Biologics Standards
July 1967 through June 1968

A. Research Activities

I. Virus Detection: Major techniques under development this year for detecting latent adventitious viruses in biologicals are: (a) electron microscopy, (b) fluorescence microscopy, (c) precipitin reactions and (d) cytochemical reactions. Modification of these techniques have been designed to detect samll concentrations of viruses or viral antigens, whether the agents are already known or not. Cell systems studied intensively for the incidence of contaminating viruses include primary canine and primary simian cells. These studies provide new data concerning the incidence of adventitious viruses in vaccine-related cell types, the identity of the agents isolated and the effectiveness of the detection systems as we and others apply them.

II. Virus Characterization: Viruses studied this year include adeno-associated viruses, infectious canine hepatitis virus, rubella virus, monkey hemorrhagic disease virus, an unidentified canine agent (recently isolated) and an unidentified simian agent (also recently isolated). Studies have been designed to correlate the physical and biological properties of these viruses.

III. Analytical Chemistry: Methods for chemical analysis of biological products developed or evaluated by the group under the supervision of Dr. Jerome J. Helprin included a comparison of electrolytic and gravimetric methods for analysis of moisture in dried products, and determination of mercurial preservatives and alum or aluminum phosphate in biologicals by atomic absorption spectrophotometry. Additional studies were concerned with the development of a method to measure the amount of silicone lubricant washed from disposable syringes with water, the amount of ether and alcohol remaining in Rickettsial vaccines after manufacture, and a collaborative study with Dr. Hardegree (LBP) concerning the effect of a bacterial enzyme on the fatty acid composition of Arlacel. Members of the analytical chemistry group have also continued to obtain analytical data on a variety of biological products to serve as a reference background for the chemical control of biological products.

B. Control Activities

During the calendar year 1967, chemical analyses were performed on 50 samples of biological products prepared by 13 domestic commercial establishments. Nine different biological products were analyzed by as many as 12 methods out of a repertoire of 20 analytical procedures. The results obtained were added to our collection of data on the chemical composition of biologicals.

Of the 50 samples, 9 were obtained from DBS stocks of excess samples and were analyzed as controls or to supplement other data, 2 samples were analyzed in conjunction with a complaint which had been referred to DBS, 3 samples were obtained from the annual inspection program, 12 samples were analyzed in a collaborative study with Lederle Laboratories, 3 samples were purchased, and 21 samples were analyzed on request from other laboratories in DBS. The samples analyzed were as follows:

1. Bacterial vaccines -1 sample- typhoid.

2. Viral vaccines -7 samples- influenza and mumps.

3. Rickettsial vaccines -16 samples- typhus fever and Rocky Mountain Spotted Fever.

4. Antisera and antitoxins -2 samples- immune globulin and tetanus antitoxin.

5. Polyvalent vaccines -5 samples- DPT.

6. Allergenic extracts -17 samples- various types

7. Miscellaneous -2 samples.

In addition, 138 samples of dried biologicals were analyzed at the request of LCA, in order to assist them in obtaining optimum performance from a newly installed freeze-drying apparatus.

A precision refractometer was placed in service and refractive indices were obtained on a large variety of biological products. This fundamental physical measurement should be of value in the chemical control of biologicals, and has been added to our armamentarium of methods and equipment devoted to that purpose.

A specific gravity balance was also placed in service; this permits another fundamental physical measurement to be made.

C. Contract Activities

Melpar, Inc., through a contract which began last May, has provided a service to DBS for detecting adventitious contaminants in cell cultures by fluorescence microscopy. The two technicians working on this project, who were trained initially in techniques in LBB, maintain a close liaison with LBB through the Project Officer, Dr. K. O. Smith, and the Assistant Project Officer, Mr. J. T. Thiel. The effect of this contract is to provide an extension to our own laboratory, since the technicians are supervised directly by the Project Officer.

Serial No. DBS 157
1. Biophysics and Biochemistry
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Search for infectious agents in canine cell cultures by fluorescence microscopy; their identification by standard virological techniques and by electron microscopy.

Previous Serial Number: none

Principal Investigator: K. O. Smith, Ph.D.

Other Investigators: J. F. Thiel
G. D. Clark
J. T. Newman (Melpar, Inc.)
K. J. Dunn (Melpar, Inc.)

Cooperating Unit: Melpar, Inc.

Man Years

Total: 2.1
Professional: 0.4
Others: 1.7

Project Description:

Objectives: Various cell types which are currently used for vaccine production were considered for adventitious agent examination. Canine kidney cells were selected for study because of recent reports that viral agents could be readily isolated from dog organs, because not very much is known about canine adventitious agents and because live measles vaccine is made in canine kidney cells. Street dogs were selected because of the need to obtain substantial numbers of positive cultures for evaluating the fluorescence antibody screening technique. It was hoped that (a) useful information could be obtained concerning the incidence of canine agents in kidney cells and (b) the new fluorescence antibody screening technique could be evaluated in a large scale study.

Neither cytopathic effects nor electron microscopic fine structure were criteria for primary recognition of infectious agents in this study. Rather, primary recognition depended upon antigen production in infected cells. Only two assumptions were made in screening primary cell cultures for agents:

(1) antigen would be produced in individually infected cells in sufficient quantities to be recognizable by the indirect Coons reaction and (2) antibody to the agent would be produced by the animal (or companion animals) serving as the source of cells. Identification of the agents was then accomplished by conventional virological techniques and by electron microscopy.

Major Findings: (1) Thirty-three different dogs (street dogs from Lone Trail Kennels) were sacrificed, and kidney cell cultures made from each animal. Serum was collected from each dog and saved for testing later. Some primary cells were frozen in liquid nitrogen for later reference. Other cells were maintained for 3-4 weeks at 36°C, fixed, treated with the donor animal's own serum, pooled canine and globulin and a battery of other dog sera known to be reactive against certain canine viruses. This was followed by treatment with fluorescein conjugated anti-canine globulin. Twenty-two of the thirty-three different primary cell cultures were strongly reactive by fluorescence microscopy. The fluorescence was usually cytoplasmic and sometimes occurred in syncytial foci, which were similar to those seen in control cultures infected with canine distemper virus.

(2) A second series of twenty-five different dogs from the same source were studied in a similar way, except that (a) cells were held for 4-5 weeks in roller drums, (b) over 10 selected sera were used on each cell line, in addition to pooled canine globulin and the dog's own serum, (c) scrapings from 4-5 week-old infected cultures (positive by fluorescence microscopy) were frozen at -70°C, (d) excess kidney tissue was frozen at -70°C and (e) positive cultures were studied further in order to identify as many agents as possible. Of 25 primary cell cultures in this series, 16 were clearly positive by fluorescence microscopy. Of these 16 positive cultures, 10 were found by neutralization to contain distemper virus and 2 contained infectious canine hepatitis virus. Morphologic confirmation was obtained by applying electron microscopy. Four agents are as yet unidentified and will require further study.

A useful by-product of these studies was a large quantity of primary cells apparently free of viral agents. These cells, stored in liquid nitrogen, are now being used by us and others (Cornell University and Illinois Institute of Technology) for studies on canine viral agents.

Four different sets of kidney tissue (stored frozen at -70°C), which were found by the above procedures to be most heavily contaminated with distemper virus, were selected for further study. These tissues were thawed, minced, ground to a paste, suspended at 10% by weight and inoculated at various dilutions on virus-free primary canine kidney cells. No virus could be obtained from any of the four kidneys by this technique, suggesting that isolation of distemper virus from infected kidenys is best done by culturing viable cells from the kidneys. We conclude, therefore, that this is the method of choice for isolating latent agents which occur in low concentrations in canine kidneys.

The serum from each dog was tested for antibody to distemper virus, infectious canine hepatitis virus and reovirus type 1. The most interesting finding was that isolation of distemper virus from a dog's kidney

seemed to be correlated with absence of detectable neutralizing antibody to the agent. Since few, if any, of the dogs (all adults) appeared to suffer from acute distemper at the time of sacrifice, it appears that distemper virus might remain latent within the kidneys of dogs long after they have experienced the disease or have been vaccinated with live attenuated virus (neutralizing antibody is known to decrease below recognizable levels in vaccinated dogs, requiring periodic booster inoculations).

From these studies we conclude that the indirect fluorescence antibody screening technique is practical to apply on a large scale and will reveal latent agents not recognizable by simply inoculating extracts from organs. About two-thirds of primary street dog cell cultures were infected with an adventitious agent. The two agents most frequently encountered were distemper and infectious hepatitis virus. Four agents still remain to be identified. There appears to be little advantage in holding cells longer than three weeks before examination. Distemper virus grew very slowly, produced little CPE and could not have been detected easily without fluorescence microscopy.

Extremely high titered anti-canine globulin (obtained from burro) was furnished by the Laboratory of Blood and Blood Products. The globulin from this valuable serum was conjugated with fluorescein isothiocyanate, purified by column chromatography and tested in our laboratory (LBB). Efforts are being made to further hyperimmunize the burro and collect sufficient antiserum for any future needs.

Significance to Bio-Medical Research and the Program of the Division: Information concerning the incidence, identity and characteristics of adventitious agents occurring in primary canine kidney cells, a cell type used in live vaccine production, is important to the Division.

Proposed Course of the Project: The project will be continued at a reduced level.

Honors and Awards: none

Publications:

Smith, K. O., Thiel, J. F., Newman, J. T. and Dunn, K. J.: Search for infectious agents in cell cultures by fluorescence microscopy. Neurology, 18: 165-167, 1968.

Thiel, J. F., Smith, K. O., Newman, J. T., Dunn, K. J. and Trousdale, M. D.: Isolation and identification of adventitious agents from primary cell cultures of dogs. Bact. Proceedings, 161, 1968.

Serial No. DBS 156
1. Biophysics and Biochemistry
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Search for adventitious agents in primary simian cell cultures by fluorescence microscopy; their identification by standard virological techniques and by electron microscopy.

Previous Serial Number:

Principal Investigator: K. O. Smith, Ph.D.

Other Investigators: J. F. Thiel
M. D. Trousdale
G. D. Clark
J. T. Newman (Melpar, Inc.)
K. J. Dunn (Melpar, Inc.)
H. C. Orr, Ph.D.

Cooperating Units: Melpar, Inc.
Laboratory of Virology and Rickettsiology

Man Years

Total: 2.4
Professional: 0.3
Others: 2.1

Project Description:

Objectives: Primary simian kidney cells were chosen as another cell type for studies concerning the incidence of adventitious agents in cell cultures. Simian cells were selected because of their current importance as a substrate for vaccine production, as well as their wide usage in basic virus research and in standard virus titrations. Our major objectives were: (1) to study commercially available primary simian kidney cells for incidence of agents; (2) to study the incidence of adventitious agents in cultures obtained from licensed manufacturers of viral vaccines; and (3) to compare the results we obtain by fluorescence microscopy with those obtained by the manufacturers, who use the adventitious virus detection systems now prescribed by law. The methods we employed in these studies were almost identical to those we employed in the canine kidney studies.

Major Findings: (1) Of over 150 different lots of primary simian cells obtained from four commercial sources (not vaccine manufacturers), over 90% contained one or more viral agents. Many cultures contained two different viral agents. African Green monkey cell cultures had as high an incidence of adventitious agents as those derived from Rhesus monkeys. Although cytopathic effects were obvious in a majority of positive cultures, some positive cultures showed little, if any, overt signs of infection except for brilliant fluorescence when treated with antiserum and viewed in the fluorescence microscope Many of these agents could be provisionally classified as either cubical or helical in symmetry by electron microscopy. These examinations revealed cubical symmetry particles of 45 and 107 mμ diameter, and helical symmetry viruses which probably belong in the para-influenza group. Further classification and identification of these agents, which are frozen, will require the application of standard viral techniques, including neutralization with specific antisera. It is of interest, however, that only one herpesvirus has been recognized and no adenoviruses have yet been observed. The majority of agents appear to be SV-40, foamy agent or SV-5. At least one agent is unlike any of the viruses commonly encountered, and this may be a new agent. It replicates very slowly, produces minimal CPE and causes an unusual nuclear inclusion.

(2) Four vaccine manufacturers are currently sending primary African Green monkey cell cultures to LBB on a regular schedule for fluorescence antibody study. This work, which is well under way, will make possible an evaluation of the incidence and type of adventitious agents occurring in cell lots similar to those actually used in viral vaccine production and will allow us to compare the sensitivity of legally required testing procedures with the fluorescence antibody techniques which we now employ. The results of these studies are considered confidential unless indicated otherwise by the manufacturers supplying the cells.

(3) The primary simian cell studies indicate that fluorescence microscopy by the indirect Coons method, formerly used on a small scale, can be practically applied to large numbers of cell cultures as a means of detecting adventitious agents. Electron microscopy of positive samples was useful for making a rapid presumptive identification of new isolates. Over 1500 fluorescence antibody preparations were made of over 150 simian cell cultures within a period of about four months. About two-thirds of the viral agents found were classified morphologically by examination in the electron microscope. A most useful observation derived from electron microscopy of these specimens was that a high percentage of fluorescence antibody positive cultures contained more than one agent.

Significance to Bio-Medical Research and the Program of the Division: Study of primary simian cell cultures for adventitious agents, their characteristics, and the study of methods to detect these agents are important to the Division.

Proposed Course of Project: This project will be continued.

Honors and Awards: none

Publications: none

Serial No. DBS 86
1. Biophysics and Biochemistry
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Detection and characterization of viruses

Previous Serial Number: Same

Principal Investigator: K. O. Smith, Ph.D.

Other Investigators:
W. D. Gehle
M. D. Trousdale
J. F. Thiel
T. E. Hobbins, M. D.
S. Sundborg
R. L. Kirschstein, M.D.
E. A. Peters
N. M. Tauraso, M.D.
H. Montes de Oca, M.D.

Cooperating Units: Laboratory of Pathology
Laboratory of Viral Immunology
Laboratory of Virology and Rickettsiology

Man Years

Total: 3.5
Professional: 1.3
Others: 2.2

Project Description:

Objectives: (1) To correlate physical and biological properties of viruses.

(2) To develop more specific and more sensitive methods for detecting, identifying and classifying viruses. Two systems being studied intensively are precipitin reactions and a method for pelleting small quantities of virus for thin sectioning-electron microscopy.

(3) To study the structure and immunologic reactions of viruses at the molecular level by electron microscopy; special attention is being given to the properties of adeno-associated viruses (AAV) and their interactions with adenoviruses.

(4) To study the in vitro interaction of recently isolated canine and simian cell adventitious agents with human cells. These studies involve primarily abortive virus-cell interactions, such as infectious canine hepatitis virus in human cells.

Methods Employed: High resolution electron microscopy, virus particle counting, complement fixation reactions, precipitin reactions, agglutination reactions, fluorescence microscopy, cell cloning and virus infectivity titrations in tissue cultures are being used to study selected virus-cell systems.

Major Findings: (1) Electron microscopy and fluorescence microscopy of rubella virus (with Dr. Thomas E. Hobbins, LVI). The morphology of rubella virus was studied by electron microscopy, correlating particle counts of purified preparations with infectivity and hemagglutinin (HA) titers. Precise measurements of infectivity were obtained by a fluorescent plaque assay technique. RK_{13} cells were fixed with methanol 72 hours after infection, treated with convalescent human serum and stained with fluorescein conjugated anti-human globulin. Foci consisting of groups of cells exhibiting granular cytoplasmic fluorescence were counted. Counts were inversely proportional to virus dilution, highly reproducible and correlated well with conventional titration results. Rubella virus centrifuged over a preformed CsCl gradient for 90 minutes at 75,000 x G gave a sharp, opaque band at density 1.22 - 1.23. Recovery of infectivity from crude suspensions after gradient centrifugation was almost complete. Infectivity and HA were concentrated in the band. Electron microscopic examination of the band showed structured particles 42-67 mμ in diameter. When fixed in osmium and negatively stained, particles appeared spherical, average 55 mμ in diameter and showed a spicule substructure on their outer surfaces. Particle-infectivity ratios were in the region of 250:1 to 500:1. These quantitative experiments, which correlated physical with biological properties of rubella virus, give strong support to the conclusion that the infectious, HA active particle has an average diameter of 55 mμ and physically resembles myxoviruses more than the paramyxoviruses.

(2) Persistence of adeno-associated virus in human cells without adenovirus potentiation (with Warren Gehle). Cell cultures initially containing about 1/3 AAV-infected cells were subcultured through several generations and studied to determine with what efficiency they retained the ability to produce AAV when super-infected with adenovirus. No substanial loss or increase in numbers of AAV-infected cells was observed during a growth period associated with a 33-fold increase in total cells. The relative numbers of infected cells decreased at a rate approximating the rate of total cell growth. Infected cells divided to produce both infected and non-infected cells. An infected cell sometimes gave rise to more than one infected daughter cell. The low frequency of this phenomenon, however, compared with the relatively high frequency of uninfected cell division, soon resulted in a "dilution" of infected cells by uninfected cells. AAV-antibody failed to neutralize AAV or "cure" infected cells, indicating an intracellular location of the virus. Cell disruption caused an almost complete loss of AAV, which suggests that the virus exists within the cell in a sub-viral form. Thus latent AAV infection of human cells resembles the lysogenic state in bacteria in a number of respects: (1) the viral genome is preserved with high

efficiency in the infected cell without potentiation; (2) the virus, while in this state, is difficult if not impossible to detect directly; (3) the virus is "triggered" into a replicative cycle under certain specific conditions (adenovirus potentiation); (4) demonstration of virus infectivity depends upon the preservation of the viable host cell; and (5) viral genome is passed along to daughter cells (rarely, in the case of AAV).

(3) Inhibition of adenovirus 12 oncogenicity by adeno-associated virus (with Dr. Ruth L. Kirschstein and Elizabeth Peters). AAV partially inhibits oncogenesis of adenovirus 12 when the two are inoculated together. Sixty percent of newborn hamsters inoculated with adenovirus 12 alone developed subcutaneous undifferentiated malignant tumors. Only 19% of animals inoculated with identical amounts of adenovirus 12 with AAV developed tumors. These results indicated that it is important to determine whether adenovirus strains used in oncogenicity experiments are contaminated with AAV (see Laboratory of Pathology annual report for more complete description of this work).

(4) Studies on infectious canine hepatitis virus (ICHV) replication in human cell cultures (with Warren Gehle). The demonstrated incidence of ICHV as an adventitious virus in some primary canine kidney cells, a cell type which is used in the production of live measles vaccine, led us to study the interaction of this virus with human cells in vitro. Within 48 hours after infection of human amnion cell cultures, large quantities of complement fixing (CF) antigen were produced, although infectious virus yields were small. Serial passage of ICHV in human amnion cells, with harvests every 48 hours, resulted in a rapid decrease in infectious virus. There was, however, a relatively slow decrease in CF antigen production on serial passage. If the amount of input infectious virus is taken as a reference, calculations indicate that the ratio of CF antigen per infectious unit increased with each serial passage. This phenomenon was most noticeable after the first serial passage in HA cultures. The mechanism of this phenomenon is as yet unknown, but our data is compatible with the hypothesis that a transmissable, defective ICHV particle is capable of inducing antigen formation in human cells without producing fully infective virus. Double diffusion agar precipitin studies indicate that the antigens produced in human cells following ICHV infection are similar (if not identical) to those produced in canine cells. The sequence of events observed in ICHV infected human cells has been compared in detail with that seen in canine cells.

(5) Design and study of a device for pelleting viruses or virus-infected cells so as to permit ultra-thin sectioning. A device has been designed for pelleting extremely minute masses of virus into a pointed polyethylene tube so that the concentrated virus can be fixed, plastic imbedded and sectioned in place, without pellet manipulation. Crude or purified preparations containing insufficient quantities of virus to give visible pellets have been successfully examined by electron microscopy. The purpose in designing and studying this device was to allow electron microscopic examination of biologicals which are suspected of containing small concentrations of adventitious viruses. This physical technique for detecting adventitious agents might have special application in the examination of killed viral vaccines (or in materials where biological detection is not possible). The

limits of this method's sensitivity are being studied, using vaccinia, influenza, and adenoviruses as models. Preliminary concentration and purification of crude viruses increases the sensitivity by a large factor. The device has been borrowed and used by workers in the National Cancer Institute for pelleting tumorigenic viruses, so it may find general use as a convenient means of pelleting viruses for thin-section electron microscopy.

(6) Physical characterization of an agent causing hemorrhagic disease in monkeys (with Dr. N. Tauraso, LVR). Electron microscopic studies of purified preparations indicate that this agent has an average diameter of 41 mμ, a modal diameter of 35 mμ and is somewhat irregular in structure. Two-thirds of purified particles had a diameter of 31-45 mμ. There was no apparent morphologic symmetry and little evidence of spicule substructure. The agent banded somewhat diffusely between densities of 1.18 and 1.23 in CsCl. Studies correlating particle numbers with infectivity are in progress.

(7) Enhancement of precipitin reaction sensitivity by centrifugation. We found earlier that viral antigen-antibody complexes could be sedimented upon dense "cushions" of CsCl solution so as to make precipitin bands more easily visualized. Further study of this technique with adenoviruses and adeno-associated viruses indicate that it may have value in detecting very low concentrations of these and other adventitious agents. As little as 10-15 micrograms of purified globulin can be detected by this method. An effort is being made to determine the practical limits of this technique for detecting adventitious agents known to occur in cell systems used for viral vaccines. Such a method would be of special value if applied to materials having no infectious or physically intact virus particles, since the only requirement would be that the material contain reactive antigens.

(8) Sensitivity of various stable cell lines to viruses (with Melvin D. Trousdale). Large differences are commonly observed between different cell lines in their ability to support the replication of viruses. Few clonal cell lines have been selected for growth of specific viruses. We are currently attempting (a) to determine the sensitivity of a variety of human stable uncloned cell lines to adenovirus type 12 and (b) to select clones from favorable cell lines which are especially sensitive to this virus. Preliminary work has shown that some clonal lines of human amnion cells are much more sensitive to adenovirus infection than others. Attempts are being made to determine the reason for these sensitivity differences by correlating relative sensitivity with morphological and cytochemical differences. Using adenovirus 12 as a model for selecting sensitive clonal cell lines, attempts will be made to select lines more suitable for the production and titration of other viruses. Availability of highly sensitive cell lines will facilitate detection and study of adventitious viruses, as well as the titration of viruses used in vaccines.

(9) Design and study of a device for spreading very small volumes of virus inoculum or antisera over cell monolayers (with Sarah Sundborg). A shaker device giving a very rapid lateral motion each 30 seconds was designed in consultation with the NIH Instrument Shop. Quantitative studies with different volumes of adenovirus inoculum showed that as little as 0.05 ml inoculum was evenly distributed over cell monolayers if adsorption was accomplished on the shaker. Distribution of virus was poor if adsorption

was done without the device. A total of three of these shaker devices has been built by the Instrument Shop and Lawshe Instrument Company is now manufacturing it for sale (with the permission of the Government). It can be used for either virus adsorption or for fluorescence antibody staining where very small volumes are required (small volumes of either virus or conjugated antibody are much more efficiently adsorbed to the cell monolayers than are large volumes).

Significance to Bio-Medical Research and the Program of the Division: Basic studies of animal viruses, as well as the development of techniques for recognizing latent or adventitious agents, provide information and methodology required for the regulation of viral vaccines.

Proposed Course of Project: This project will continue.

Honors and Awards: none

Publications:

Smith, K. O. and Thiel, J. F.: Adeno-associated virus studies employing a fluorescent focus assay technique. Proc. Soc. Exper. Biol. Med., 125: 887, 1967.

Thiel, J. F. and Smith, K. O.: Fluorescent focus assay of viruses on cell monolayers in plastic petri plates. Proc. Soc. Exper. Biol. Med., 125: 892, 1967.

Smith, K. O.: Detection of Adeno-associated viruses (AAV). J. Nat. Cancer Inst., in press.

Smith, K. O., Gehle, W. D. and Montes de Oca, H.: Persistence of adeno-associated virus in human amnion cells without adenovirus potentiation. Bact. Proc., 153, 1968.

Gehle, W. D. and Smith, K. O.: Studies on infectious canine hepatitis virus in human cell cultures. Bact. Proc., 160, 1968.

Hobbins, T. E. and Smith, K. O.: Electron microscopy and fluorescence microscopy of rubella virus. Bact. Proc., 181, 1968.

Kirschstein, R. L., Smith, K. O. and Peters, E. A.: Inhibition of adenovirus 12 oncogenicity by adeno-associated virus. Proc. Soc. Exper. Biol. Med., in press.

Serial No. DBS 29
1. Biophysics and Biochemistry
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Development of methods for the chemical analysis of biological products.

Previous Serial Number: SAME

Principal Investigator: Jerome J. Helprin, Ph.D.

Other Investigators: Harry P. Marshall
J. Richard Miller
Clarence A. Robinson
Joseph W. West

Cooperating Units: None

Man Years

Total: 5.0
Professional: 1.0
Others: 4.0

Project Description:

Objectives: To develop new methods of chemical analysis of biological products or to modify existing methods, thus facilitating the study of chemical problems in the control of and research on biologicals.

Methods Employed: Standard methods of chemical analysis are used, evaluated, or modified as required for the material being analyzed, and new methods are developed.

Major Findings: (1) Work has continued on the evaluation of a method developed in LBB to determine the mercurial preservative thimerosal (Merthiolate, Lilly) in biologicals. A collaborative study is now under way with a manufacturer of biologicals to analyze samples of biologicals by this method and by a traditional wet chemical method. The results have been in close agreement for most products tested so far. Some improvements in equipment only now becoming commercially available promise to make the method even more sensitive, precise, accurate, and rapid.

(2) We have obtained the necessary equipment and trained a technician so that we are now able to analyze dried biologicals by both the electrolytic method and the traditional gravimetric method, and have set up a program to correlate the results obtained by both methods.

(3) A study to measure the amount of silicone lubricant washed from disposable syringes was completed. A manuscript is being prepared to describe the method which we developed to make this measurement, and also the trace amounts of lubricant which we found.

(4) The gas chromatograph and infrared spectrophotometer were employed to measure the amounts of residual solvents (ether and alcohol) in typhus fever and Rocky Mountain Spotted Fever vaccines. A fifty-fold difference in the alcohol content was found between the typhus fever vaccines of different manufacturers. Unfortunately, we were not successful in obtaining collaboration within DBS to determine if this difference has any biological effect.

(5) A collaborative study with Dr. Hardegree (LBP) has been initiated to study the effect of a bacterial enzyme on the fatty acid composition of Arlacel, used as an adjuvant in certain vaccines. This work was initiated too recently to permit comment as to the results, although preliminary work with the gas chromatograph has gone very smoothly.

(6) A study with Dr. Montes de Oca (LVR) to determine a-tocopherol (Vitamin E) in tissue culture fluids was concluded when he left DBS, but the results indicated that a more sensitive analytical method than we were using would be needed; we suggested gas chromatography.

Significance to Bio-Medical Research and the Program of the Division: The significance of this work lies in providing increased knowledge of the chemical composition of biologicals which can be of value in research, and in surveillance of these products for the protection of the public.

Proposed Course of Project: This project will be continued.

Honors and Awards: None

Publications: None

Adjuncts to OD
Contracts
Investigators
LBBP
LCA
Publ
LP
LVI
ject Inc
LVR

Annual Report of the
Laboratory of Blood and Blood Products
Division of Biologics Standards
July 1, 1967 through June 30, 1968

The primary mission of the Laboratory of Blood and Blood Products is to control the safety, purity and potency of blood and blood products. The rapidly expanding field of blood therapy and the many recent developments in medical technology have their impact on new uses for blood and its components. This is naturally reflected in the number of new product applications received and the increase in establishments wishing to be licensed as manufacturers.

During this past year, there have been several changes in key personnel. Dr. E. H. Mealey resigned as Acting Chief of the Laboratory and Chief of the Blood and Blood Derivatives Section. Dr. Sam T. Gibson replaced him as Acting Chief, assuming this responsibility in addition to those he carries as Assistant Director of the Division. In February, Dr. Pinya Cohen joined the Laboratory as Chief of the Blood and Blood Derivatives Section.

The decrease in the total number of staff members and temporary vacancies in the major supervisory positions have caused unusual demands to be made of the entire staff, and particularly of the senior staff members who served as acting incumbents. The exemplary manner in which everyone responded to increased workloads and responsibilities was noted and appreciated.

I. Intramural Research Activities: Laboratory of Blood and Blood Products includes studies of the stability of blood products designed to yield information needed in developing acceptable storage conditions and dating periods for materials derived from blood. Proteins of the clotting and fibrinolytic systems are targets of active investigation to provide a basis for improved control tests for these products. Increasing attention has been devoted to the stability, characteristics, preparation, and assay of proteins with antibody activity in view of the great interest in immunologically active products derived from blood.

II. Collaborative Research and Contracts: To supplement the research activities of the Division and to yield data bearing on the development of regulatory standards, four research contracts for which members of the Laboratory are senior project officers are in force. Two of these are concerned with the effects of storage conditions on plastic blood containers (Baylor University, University of Texas) and two are concerned with the study of the carcinogenic potential of chemicals used in biological preparations (Mason Research Institute, Wisconsin Alumni Research Foundation).

III. Other Than Research Activities: Of the 220 licensed manufacturers, 76 percent manufacture one or more products for which this Laboratory has primary responsibility. Currently eight qualified inspectors

and four inspector trainees comprise the laboratory personnel who are involved in this control activity. In fulfillment of the requirement to inspect licensed establishments at least once a year, 278 man days were spent in visiting 331 locations, representing 130 licensees. (See Table I.)

The other major control function conducted by the Laboratory is the testing of samples submitted for release, licensure or as a result of an inspection. A total of 24,312 physical, chemical or immunological tests were performed on 3,894 lots of products to determine their safety, purity and potency. (Table II summarizes these tests.)

An exceptionally large number of license applications were submitted for approval. Fifteen manufacturers requested establishment licenses to produce blood and blood products. Sixty product license applications and 41 amendments to existing applications were submitted. Nine new establishments were licensed, 26 product licenses were issued and 33 amendments were approved. (See Table III.)

Applications for new products not previously approved by the Division require the combined concerted efforts of both applicant and the Laboratory in order to meet the requirements for licensure. Two of these products were approved this year: Rh_o (D) Immune Globulin (Human) and Citrate Phosphate Dextrose Whole Blood (Human). CPD anticoagulant marks the first addition to the list of approved anticoagulants for whole blood since heparin was included in 1961. Ten of the applications currently pending are for previously unlicensed products.

Work on the preparation of current Tentative Technical Standards continues. Standards for Red Blood Cells (Human), including requirements for frozen blood cells, and for Single Donor Plasma (Human), including criteria when plasmapheresis is the method of plasma collection were completed and forwarded to be promulgated as Additional Standards. These have been distributed to licensees for comment. Tentative Technical Standards for Rh_o (D) Immune Globulin (Human) have also been prepared and circulated for informal comment.

Four hundred sixty-seven groups of labels and circulars for blood products were reviewed for compliance with Regulations. The change in the name of Citrated Whole Blood (Human) to ACD Whole Blood (Human) was partially responsible for the large number of revised labels.

The Laboratory is grateful to the members of the donor panel who have been so cooperative in providing blood samples as a source of special blood phenotypes. Nine hundred fifteen blood samples were drawn by the Laboratory staff for the control tests of licensed products.

Staff in-service training continues, with monthly meetings to discuss research projects currently in progress and biweekly seminars to keep the inspecting staff aware of new trends and regulations. Experts in the field of blood banking invited to participate in this training, provide an important source of information on developing research and practical applications of products controlled by the Laboratory.

Whenever possible, staff members are encouraged to enroll in the training opportunities provided at NIH, and 9 staff members have completed 11 of the courses of instruction offered. In addition, special technical seminars of particular interest to areas of assigned responsibilities were attended.

Summaries of Certain Control Activities
Laboratory of Blood and Blood Products
Division of Biologics Standards
July 1, 1967 through June 30, 1968

TABLE I

CONTROL ACTIVITIES REQUIRING TRAVEL STATUS

Type of Activity	Locations Inspected	Contracts in Effect	Man Days of Travel
Annual Inspections	331		278
Supportive of Contracts		4	6
			284

Summaries of Certain Control Activities
Laboratory of Blood and Blood Products
Division of Biologics Standards
April 1, 1967 through March 31, 1968

TABLE II

CONTROL TESTING OF BLOOD AND BLOOD PRODUCTS

Type of Product	Number of Lots Tested				Number of Tests			
	Inspection Sample	For License	For Release	TOTAL	Purity	Potency	Safety	TOTAL
Blood Derivatives		20	1,430	1,450	1,301	256	1,727	3,284
Diagnostic Serums	124	40	1,083	1,247	7,567	6,256		13,823
Reagent Red Blood Cells	42	5		47		2,088		2,088
Whole Blood, Plasma Packed Red Blood Cells	516	59	575	1,150			5,117	5,117
	682	124	3,088	3,894	8,868	8,600	6,844	24,312

Status of License Applications
Laboratory of Blood and Blood Products
Division of Biologics Standards
July 1, 1967 through April 30, 1968

TABLE III

	Establishment Applications	Product Applications	Amendments	Total
No. Pending, Close FY 67	7	37	28	72
No. Received FY 68	15	60	41	116
No. Recommended	9	26	33	68
No. Withdrawn or Inactive	2	3	5	10
No. Pending	11	68	31	110

CONTRACT NARRATIVE
LABORATORY OF BLOOD AND BLOOD PRODUCTS
DIVISION OF BIOLOGICS STANDARDS
July 1, 1967 through June 30, 1968

BAYLOR UNIVERSITY (PH43-65-1046)

UNIVERSITY OF TEXAS (PH43-68-97)

Title: Effects of Storage Conditions On Plastic Blood Containers

Contractors' Project Directors: Sol Haberman, Ph. D.*
Wallace L. Guess, Ph. D.

Project Officers (DBS): Madge L. Crouch
Clarence F. Szwed

Objectives: To determine the effects of storage time and temperature on plastic blood containers of different manufacturers, in order to evaluate the necessity for specifying dating periods and storage conditions for these containers.

Methods Employed: Extracts of the ACD anticoagulant and samples of stored whole blood and plasma were subjected to physical-chemical and biological tests. The toxicity of the extracts was studied through intravenous injection of mice and the intradermal injection of rabbits. In addition, in vivo cell survival studies and immunological reactivity tests on stored erthryocytes and plasma were conducted. These studies are being conducted as a cooperative project between these two contractors.

Major Findings: ACD anticoagulant stored in plastic containers was found to contain varying quantities of leached substances, largely composed of epoxydized soya oil and phthalic acid. The extractant used influences the amount of extractive material, and some standardization of these solvents is essential if meaningful comparisons are to be made by investigators. Preliminary tests of stored contents seem to suggest that products stored in plastic containers may have teratogenic effects, when injected into developing chick embryos. Hemagglutination inhibitor for the rubella test, not demonstrable when glass containers are used, and some increased fragility of red cells stored for the maximum dating period in plastic containers were demonstrated. Definite quantitative effects of storage time and temperatures cannot be determined until the completion of the contracts.

*Deceased April 16, 1968

Significance to DBS Program and Bio-Medical Research: Because of the wide-scale use of plastic containers for blood and blood products, the definitive data being compiled are useful in determining if specifications on formulations, dating, and storage conditions are indicated in order to assure the safety, purity and potency of the contents.

Proposed Course of Project: These contracts will continue through August 27, 1968 and October 22, 1968, respectively.

Date Contract Initiated: Baylor University-June 28, 1965
University of Texas-March 30, 1964

Current Annual Level: Baylor University-$48,986.00 additional funds, making the total contract amount $276,980.00. University of Texas-$33,430.00 additional funds, making the total contract amount $136,530.00.

CONTRACT NARRATIVE
LABORATORY OF BLOOD AND BLOOD PRODUCTS
DIVISION OF BIOLOGICS STANDARDS
July 1, 1967 through June 30, 1968

MASON RESEARCH INSTITUTE (PH43-67-676)

WISCONSIN ALUMNI RESEARCH FOUNDATION (PH43-67-684)

Title: A Study of Carcinogenic Potential of Seven Chemicals Used in Biological Preparations

Contractor's Project Directors: Marcus M. Mason D.V.M.
Philip H. Derse

Project Officers (DBS): Donald L. Powell
Stanley H. Singer, M.D.

Objectives: To test the oncogenic potential and co-carcinogenic activity of the following seven compounds found in biological products as preservatives, inactivating agents, stabilizers, or additives: thimerosal, benzethonium chloride, parabens, phenol red, pyridine, ethylene glycol, and ethylene chlorhydrin.

Methods Employed: The injection by various routes of several species and ages of animals with the defined compounds alone and in combination with other substances.

Major Findings: None

Significance to DBS Program and Bio-Medical Research: These programs constitute part of the continuing effort to assure that various components of biological products are free from carcinogens or co-carcinogenic activity.

Proposed Course of Project: These contracts will continue for one year and one-half year, respectively.

Date Contract Initiated: February 16, 1967
Renewed: March 1, 1968

April 27, 1967
Possible Extension From: July 26, 1968

Current Annual Level: $71,150.00 additional funds for total contract of $135,150.00.

$50,283.00

Serial No. (NIH-DBS) 33
1. Blood & Blood Products
2. Blood & Blood Derivatives
   Coagulation
   Diagnostic Reagents
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Preparation, Characterization, Stability and Potency of Blood Products

Previous Serial Number: Same

Principal Investigator: John S. Finlayson, Ph. D.

Other Investigators: Barbara Lynne Ehrenreich
Brenda Flam, B. S.*
Catherine Knapp, B. S.*
Herman Hudson
Elizabeth B. Paul, A. B.
Joan Roelands, B. A.
Peter C. Stang, A. B.
Mary C. Strott
Clarence F. Szwed, M. S.
Donna Williams, B. S.
Mildred L. Woodward, B. S.
Andrew M. Young, M. S.

Cooperating Units: Laboratory of Biophysics and Biochemistry (Dr. Kendall O. Smith); Animal Farm Center (Dr. Raymond D. Zinn); Division of Research Services (Dr. Stephen Potkay)

Man Years:
Total: 5.0
Professional: 2.4
Other: 2.6

Patient Days: None

Project Description:

Section I. Stability of Blood Products

Objectives: To determine the changes in properties of blood proteins which occur during processing, storage and related treatment.

*Resigned

Methods Employed: a) Studies on the effects of heating on Immune Serum Globulin (Human) have been continued. Both the immediate effects on physical properties and long-term effects on storage stability have been investigated. (Finlayson, Young & Hudson)

b) The study of the stability of Blood Grouping Sera at different storage temperatures has been continued, utilizing the methods routinely employed for the control of such sera. In addition, a similar study on stability of certain Anti-Rh Typing Sera was begun. (Strott)

Major Findings: a) Ultracentrifugal studies to date have indicated that the endogenous proteolytic activity of Immune Serum Globulin (Human) could be greatly reduced, but not abolished by heating at 45° C. or 50° C. prior to storage.

b) Approximately 860 tests performed during the third year of storage of the Blood Grouping Sera have, within the limits of experimental error, demonstrated almost no loss in potency. In the case of the Anti-Rh Typing Sera, however, complete loss of potency has been detected in some of the sera for saline tube tests.

Section II. Development of Methods and Standards

Objectives: To develop preparations for standardization of assay methods, to modify and extend existing assay techniques.

Methods Employed: a) Potential reference samples of human chorionic gonadotropin, consisting of two dried preparations, were distributed to manufacturers of Anti-Human Chorionic Gonadotropic Serum for assessment of potency. (Williams & Paul)

b) To place the Laboratory in a better position to evaluate antisera recommended for use in automated blood grouping and typing techniques, feasibility studies were undertaken utilizing the Auto-Analyzer^{R}. (Paul & Williams)

c) In view of the pressing need for control of Immune Serum Globulin (Human) of high anti-Rh_o (anti-D) titer intended for use in immunosuppression, a large portion of the staff's time was devoted to examination of this material and to developing a suitable assay for it. In addition to tube titration and related techniques, a radioimmunoassay was investigated extensively. This necessitated the development of radio-labeling techniques, procedures for separating protein-bound and non-protein-bound radioactivity, counting techniques, and computer processing of the data. (Paul, Williams, Stang & Finlayson)

d) In order to develop materials which might be used in carrying out or evaluating quantitative measurements of subclasses of immunoglobulins, isolation of purified immunoglobulins from normal human serum (as opposed to myeloma serum) was undertaken. (Roelands & Hudson)

e) In association with the NIH Farm Animal Center the program of immunization is continuing to prepare specific dog blood grouping serum as well as burro anti-canine gamma globulin and burro anti-human gamma globulin. (Strott & Szwed)

Major Findings: a) Results of testing the human chorionic gonadotropin preparation have been received and compiled, and evaluation of the findings has been undertaken.

b) It has been possible to duplicate results of published grouping and typing methods utilizing the Auto-Analyzer^{R}. Six samples from one manufacturer, recommended for use in automated testing were evaluated. Four of them proved to be satisfactory. This procedure may be of value in other immunoassays.

c) A preparation has been obtained which can be used as a reference standard for anti-Rh_o (anti-D) assays. This material has been examined and assayed by the various methods described above, as have several other preparations. Rh_o (D) positive donors have been identified whose cells are of suitable antigenicity for use in the radioimmunoassay. The radioimmunoassay with its required ancillary procedures (radio-labeling, chromatography, ultracentrifugation, counting, computerization, etc.) has been carried out successfully. However, when its demands in terms of time, cost, and staff are compared with the resultant precision, it becomes clear that a more suitable routine assay is desirable.

d) Since relatively pure human IgG had been prepared (see part e), preparation of human IgM was attempted. A preparation enriched (as evaluated by analytical ultracentrifugation) in this material was obtained and used for immunization. The resultant antiserum manifested an anti-IgM component in preliminary immunodiffusion tests.

e) Information has been obtained regarding the stability of canine blood grouping serum during storage. The antisera obtained have been widely distributed to various university medical schools and investigators for use in establishing dog blood donor colonies. The methodology developed during these studies has been made available to researchers wishing to prepare their own purified antigens and specific antisera. The burro anti-canine gamma globulin has, inter alia, been used in LBB for fluorescein conjugation detection of adventitious viruses in dog cell cultures.

Significance to Bio-Medical Research and the Program of the Division: The Laboratory of Blood and Blood Products is responsible for control of the safety, purity and potency of products derived from blood. Such surveillance involves the possession of knowledge regarding the stability of biological products as well as the availability of appropriate control test methods and technical standards. This project fulfills these needs by providing information leading to more realistic dating periods and control test methods.

Proposed Course of Project: This is a continuing project.

Honors and Awards: None

Publications: Pennington, R. M. and Gibbs, C. J., Jr.: A simplified method for preparing eastern and western equine encephalomyelitis complement fixing antigens. Proc. Soc. Exp. Biol. Med. 125: 787-789, July 1967.

Strott, M. C., Zinn, R. D. and Pennington, R. M.: The preservation of dog agglutinogens in liquid nitrogen. Vox Sang. 13: 27-28, No. 1, 1967.

Serial No. (NIH-DBS) 81
1. Blood & Blood Products
2. Coagulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Clotting and Fibrinolytic Systems

Previous Serial Number: Same

Principal Investigator: David L. Aronson, M. D.

Other Investigators: John S. Finlayson, Ph. D.
Mervin Taylor, B. S.
Herman Hudson

Cooperating Units: National Cancer Institute (Dr. J. Frederic Mushinski);
National Heart Institute (Dr. John J. Pisano)

Man Years:

Total: 3.1
Professional: 1.0
Other: 2.1

Patient Days: None

Project Description:

Objectives: To purify and characterize components of these systems and to develop an understanding of their chemistry as a basis for more useful assay procedures.

Methods Employed: a) Human thrombokinase (Factor X^a) which has been partially purified in this laboratory by fractional precipitation, chromatography, and preparative disc electrophoresis was studied in several different ways. In association with Dr. J. Frederic Mushinski, National Cancer Institute, tryptic peptide maps of human thrombokinase were prepared and compared with those of human prothrombin (Factor II). A number of phosphonate compounds were tested for possible inhibitory activity toward thrombokinase. Certain bacterial enzymes were investigated for ability to activate prothrombin and prothrombokinase. (Aronson)

b) The work on streptokinase was tentatively concluded, and the development of a viscometric assay for streptodornase, a deoxyribonuclease present in all streptokinase preparations, was undertaken. Information from this assay will be used in determining the feasibility of replacing the present NIH unit for streptodornase with the international unit. (Hudson)

c) In association with Dr. John J. Pisano, National Heart Institute, studies were made of the chemical nature of the crosslink in human fibrin polymerized by fibrin stabilizing factor (Factor XIII). These utilized enzyme digestion and peptide isolation methods developed in Dr. Pisano's laboratory and Factor XIII and/or plasminogen free fibrinogen prepared by methods developed in this laboratory. (Finlayson)

d) To develop standards required for the control of antihemophilic factor (Factor VIII), samples of freeze dried plasma and cryoprecipitate were tested for antihemophilic factor activity and the levels were compared with those of antihemophilic factor in freshly drawn plasma from a normal donor population. (Taylor)

Major Findings: a) The ability of human thrombokinase to convert prothrombin to thrombin was shown to be independent of any residual thrombi content. This converting activity is inhibited by soybean trypsin inhibitor but not by diisopropylphosphorofluoridate. Of the phosphonates tested, none inhibited thrombokinase unless used at very high concentration. Various cofactors (polylysine or calcium alone, or beef serum or phospholipid in the presence of calcium) were found to accelerate the conversion of prothrombin to thrombin by thrombokinase. Tryptic peptide maps of prothrombin and thrombokinase were completely different, showing that the latter molecule cannot be derived from the former. Bacterial thermolysin could activate both prothrombin and prothrombokinase; subtilisin could activate prothrombin.

b) The viscometric assay of streptodornase has progressed to a point at which linear plots of the data (showing reciprocal relative viscosities versus time of incubation) can be obtained. Studies are in progress to ascertain optimal substrate concentrations and to obtain slopes which yield ratios between activities of standard and commercial lots which agree with those reported by the manufacturer.

c) ε-(γ-Glutamyl) lysine was shown, by direct demonstration of the isolated dipeptide, to be the crosslink in human fibrin polymerized by human Factor XIII. The amount of crosslinking could be diminished by using a noncompetitive inhibitor of Factor XIII or by using Factor XIII free fibrinogen as starting material for the experiments.

d) The original antihemophilic factor reference plasma was found to have lost some of its potency (approximately 20 percent over a period of 4.5 years at -20° C.). A new reference plasma has been obtained, tested and found to be satisfactory as a reference standard for antihemophilic factor. Since freeze dried cryoprecipitate could be tested satisfactorily by the procedure used for assay of less purified preparations, it is anticipated that a freeze dried cryoprecipitate may also be a useful reference material.

Significance to Bio-Medical Research and the Program of the Division: Control of the purity and potency of therapeutic blood derivatives used to treat coagulation and thrombotic disorders involves detailed understanding

of the mechanisms of the clotting and fibrinolytic processes and the chemistry of the in vitro assay methods. This understanding is leading to improved validity and precision in control tests.

Proposed Course of Project: This is a continuing project.

Honors and Awards: None

Publications: Finlayson, J. S.: Chromatographic purification of fibrinogen. In Laki, K. (Ed.): Fibrinogen. New York, Marcel Dekker, Feb. 1968, pp. 39-59.

Kisker, C. T.: Effects of thrombin on Factor VIII activity: stabilization of Factor VIII by glycine ethyl ester. Thromb. Diath. Haemorrh. 17: 381-387, June 1967.

Tomar, R. H.: Streptokinase: preparation, comparison with streptococcal proteinase, and behavior as a trypsin substrate. Proc. Soc. Exp. Biol. Med. 127: 239-244, Jan. 1968.

Serial No. (NIH-DBS) 82
1. Blood & Blood Products
2. Coagulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Proteins of Body Fluids

Previous Serial Number: Same

Principal Investigator: John S. Finlayson, Ph. D.

Cooperating Units: National Cancer Institute (Dr. Michael Potter, Dr. J. Frederic Mushinski)

Man Years:

Total: 0.5
Professional: 0.3
Other: 0.2

Patient Days: None

Project Description:

Objectives: To complement available data on blood proteins with a reservior of basic information on the properties of and methods suitable for use on proteins of blood and other fluids.

Methods Employed: In association with Dr. Michael Potter, National Cancer Institute, the study of proteins of the fluids of laboratory animals was continued. In addition to studies of the physical, chemical, immunological and genetic aspects of the major urinary protein (MUP) complex of normal inbred mice, recent work was directed toward the isolation, antigenic characterization, and tryptic peptide mapping of the individual components of the complex. Isolated proteins from mice of both sexes of two inbred strains were examined.

Major Findings: Gel filtration of the nondialyzable material in the urine of normal inbred mice yielded three fractions, the first of which contained uromucoid (Tamm-Horsfall mucoprotein), the second of which contained the MUP complex, and the third of which was low in nitrogen, high in carbohydrate and had a low sedimentation coefficient (0.5 S). Tryptic mapping of the individual components of the MUP complex isolated by ion exchange chromatography indicated that electrophoretically distinguishable components 1 and 2, a genetic variant shown in this laboratory to be under the control of one genetic locus (the Mup-a locus), differed by a single peptide. Components 1 and 3, on the other hand, had a number of peptides in common plus several peptides unique to each. The peptide map of any given component did not differ between sexes or between the strains investigated.

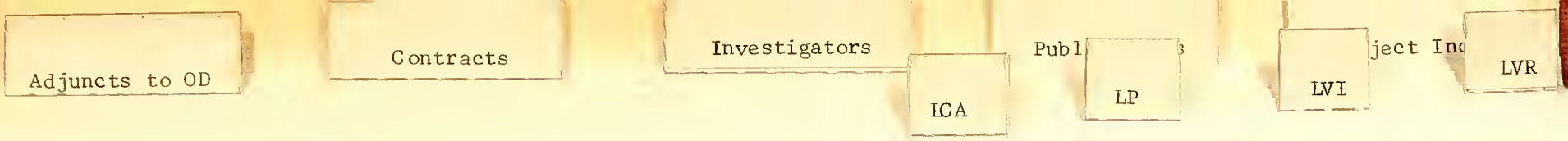
Significance to Bio-Medical Research and the Program of the Division: The control program of the Laboratory continually requires information regarding the characteristics of proteins and methods used in studying them. The basic research program described above provides the professional staff with a constantly updated pool of fundamental information (including research methodology) necessary in evaluating new products and tests and in discussions with manufacturers.

Proposed Course of Project: This is a continuing project.

Honors and Awards: None

Publications: Finlayson, J. S. and Mushinski, J. F.: Separation of subfractions of human β_2-glycoprotein I. Biochim. Biophys. Acta 147: 413-420, Dec. 1967.

Hudson, D. M., Finlayson, J. S. and Potter, M.: Linkage of one component of the major urinary protein complex of mice to the brown coat color locus. Genet. Res. 10: 195-198, Oct. 1967.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Annual Report of the
Laboratory of Control Activities
Division of Biologics Standards
July 1, 1967 through June 30, 1968

I. Mission

The Laboratory of Control Activities has prime responsibility within the Division for performing and coordinating the testing conducted on biological products to determine their conformance to standards of safety, purity and potency. The laboratory is composed of three sections: Control Testing, Pyrogens, and Reference Standards; and an administrative technical staff which performs the task of reviewing manufacturers' product protocols, and coordinating, compiling and filing the data of manufacturers submitted to the Division in connection with these control activities.

The Laboratory has the following functions:

(a) To determine whether each lot of a licensed biological product meets prescribed standards of safety, purity and potency and to exercise the necessary control actions culminating in either official release or rejection of the material intended for sale by the manufacturer in interstate commerce. Such action is based on the results of a detailed review of the manufacturers' processing and testing data as reported in manufacturing protocols, testing data compiled within the Division, and any other available source of information relating to the safety, purity or potency of the product.

(b) To develop, maintain and distribute material biological standards, references, control materials and reagents necessary for the testing of products to determine compliance with standards. A culture collection or deposit is also maintained to support the technical effort of the Division as well as to support the control activities as they affect the licensed manufacturers.

(c) To maintain under review published standards, minimum requirements, technical memoranda and other guidance issued by the Division to manufacturers concerning licensable biological products, to develop or propose revisions in regulations when indicated, and to prepare technical specifications and other regulatory procedures for new products as they are developed.

(d) To maintain a close liaison and working relationship with other laboratories of the Division and where indicated with the other Institutes of N.I.H. as well as other outside agencies to insure a continuous flow of knowledge and technical information deemed vital for the proper exercise of control measures of both those products already licensed and new products being developed for license.

(e) To participate in and perform annual inspections of designated licensed establishments in accordance with specified procedures and specific objectives as prescribed by law.

(f) To participate in and give technical support to investigations performed by the Division in connection with Public Health Service Act.

(g) To participate in the review of applications and reports of manufacturers and their products to determine eligibility for license.

(h) To participate in and give technical support to personnel training programs organized within the Division of Biologics Standards.

II. Product Testing, Protocol Review, Records

Individual lots of licensed products are controlled by the Division through an intricate system of evaluation and release utilizing data submitted by the manufacturer and that developed by the testing program of the Laboratory of Control Activities and other testing areas of the Division. All testing is directed toward the objective of assuring that products meet prescribed standards of safety, purity and potency.

Included in the purity testing of biological products are specifically those tests for pyrogenicity, residual moisture and total solids, which also serve as means for evaluating and controlling the safety and stability of such products.

The types of products controlled include antitoxins and therapeutic immune serums, blood and blood derivatives, bacterial vaccines, toxins and toxoids, multiple antigens, viral and rickettsial vaccines, diagnostic substances for dermal tests, antivenins, venoms, streptokinase-streptodornase, and allergenic extracts.

In addition, to the pyrogen test control of biological products within the Division, the Pyrogen Test Section of the Laboratory of Control Activities provides to a large measure cooperative testing services to other N.I.H. facilities such as the Clinical Center Pharmacy, Clinical Center Blood Bank, Media Department, National Institute of Allergy and Infectious Diseases, and the National Cancer Institute.

A summary of 10,144 tests performed by the laboratory for a 12 month period (April 1, 1967 through March 31, 1968) in making the determination that products meet prescribed standards is shown in Table 1.

Results of these tests served in part as a basis for concluding that products could or could not be released by the manufacturer for market. Also submitted to the laboratory for review were protocols of the manufacturer showing details of testing procedures and results of all tests performed to demonstrate that each lot of product met prescribed standards. For the year, 3,996 lots of products together with their manufacturing and testing protocols were submitted by manufacturers to the Division for consideration. Of these 3,899 lots were released, 38 lots were rejected by the DBS and an additional 59 lots were withdrawn by the manufacturers without release action being taken by the Division. A summary of these data broken down according to product groups is presented in Table 2.

TABLE 1

Samples Tested	Number of Tests Performed				
	Sterility	Safety	Potency	Purity	Total
Products for Release	3,495	796	858	862	6,011
Inspection	255	7	12	72	346
Cooperative Service	2,065	13	4	433	2,515
Reference and Standardization	282	0	964	6	1,252
Complaint	0	4	16	0	20
Totals	6,097	820	1,854	1,373	10,144

TABLE 2

Product Group	Lots Released	Lots Rejected	Lots Withdrawn	Totals
Antitoxins	16	0	0	16
Therapeutic Serums	5	0	0	5
Blood and Blood Products	1,412	2	18	1,432
Bacterial Vaccines	127	1	2	130
Toxoids and Toxins	89	0	0	89
Multiple Antigen Preparations	99	4	4	107
Viral and Rickettsial Vaccines	780	7	13	800
Diagnostic Substances for Dermal Tests	149	0	0	149
Diagnostic Substances for Laboratory Tests	1,093	24	22	1,139
Miscellaneous	129	0	0	129
Totals	3,899	38	59	3,996

In connection with release activities, the laboratory also prepared the product release actions, maintained a permanent record and filing system of all manufacturers' protocols and test data in support of each action, and maintained refrigerated storage of representative samples along with appropriate records for future reference on all biological products submitted to the Division. In all these activities the laboratory continues to re-evaluate the methods and operations relative to the establishment of more efficient control measures.

III. Material Standards

An integral part of the system of manufacture and control of biological products is the application and use of material standards. By the use of such standards potency of a product can be determined and standardized so as to give increased assurance of consistency from lot to lot. It is the function of the standards program of the laboratory to provide these standards and reference preparations so that the control program of the Division as well as manufacturers and others engaged in testing may have available the tools to calibrate and evaluate products. It is largely through use of these preparations that those portions of the biologics control regulations relating to potency can be applied.

In order to maintain an adequate supply of material references and standards it was necessary to prepare and calibrate during the past year the following lots of materials: 15 antitoxins, 3 serums, 11 vaccines, 2 toxins, and 4 skin test antigens. In addition to these materials prepared by the laboratory the following lots of materials were purchased from commercial sources and finished by the laboratory: 1 antitoxin, 9 serums, 6 vaccines, 4 toxins, and 4 virus preparations. A total of 1,046 tests was required to complete a satisfactory standardization of these materials and maintain surveillance of the stability of all preparations. These tests included such procedures as flocculation reactions, animal protection tests, animal titrations, neutralizations and assorted *in vivo* and *in vitro* tests on specific specialized materials.

The technique of freeze-drying is a key procedure applied in the preparation of standards and reference materials whenever possible in order to increase assurance of stability over long periods of storage. The following numbers of ampules of various preparations were dried during the year.

Cultures	1,106	ampules
Serums	3,515	"
Vaccines	6,035	"
Viruses	200	"
Total	10,856	ampules

The following standard and reference preparations, consisting of a total of 879 separate shipments were distributed to manufacturers, P.H.S. and state health laboratories, universities, foreign manufacturers holding U. S. licenses and qualified foreign laboratories (primarily through the World Health Organization).

Antitoxins	448
Serums	4,104
Vaccines	2,416
Toxins	548
Bacterial & Viral Cultures	391
Allergens	294
Viruses	967
Opacity Standard	39
Miscellaneous	122
Total	9,429

A culture collection of etiological agents, i.e. viral and bacterial organisms, used in the manufacturing and testing of biological products, was maintained. Approximately 100 different standards, references, and related control preparations are maintained for distribution.

IV. Related Activities

In addition to the effort outlined above, the laboratory staff also participated during the year in a number of ancillary activities directly related to control which accounted for a substantial segment of its working time.

(a) Usually one professional person (as a representative of the control laboratory interest) participated as a member of each DBS committee appointed to review, evaluate and recommend appropriate action with respect to new or revised product and establishment license applications on all biological products presented to the Division, except blood and blood derivatives in in which case the participation of the laboratory is in liaison with the Laboratory of Blood and Blood Products. Such review encompassed detailed scrutiny of manufacturer's production protocols for scientific accuracy and conformance to prescribed standards, testing of the products, inspection of manufacturing facilities, preparation of reports, etc. Sixty product licenses and ten establishment licenses were processed during the year and control testing of the products submitted in support of these applications were performed in the laboratory. Reports and recommendations were made for each action.

(b) The laboratory staff participated in sixty-nine of the on-site inspections required to be performed annually on all licensed establishments, in one pre-license inspection routinely performed on each establishment prior to recommending issuance of license and six special inspections. Approximately 108 man days of work were consumed during the year in this effort.

(c) Members of the staff participated in drafting and revising regulations and additional standards. Redrafts of technical recommendations for the manufacture of Smallpox Vaccine and Measles-Smallpox Vaccine were prepared. Drafts of recommendations for manufacture of Anthrax Vaccine, Typhoid Vaccine and Tuberculin were prepared. Final drafting of Additional Standards for Diphtheria Toxin for Schick Test was completed.

(d) The Laboratory staff participated in presenting an FDA inspectors' course on the control of radio-active biological products and pharmaceuticals.

V. Research

While the primary function of the Laboratory of Control Activities is concerned with various aspects of controlling the safety, purity and potency of biological products, a substantial research program is conducted in order to provide a sound basis for the testing program and selection of preparations for use as official standards and references. The following are summaries of some of the research conducted by Laboratory personnel during this fiscal year.

(a) Bacteroides vulgatus for Testing Fluid Thioglycollate Medium: The growth requirements of Bacteroides ovatus and B. vulgatus were compared with those of Clostridium tetani and Clostridium chauvoei in order to select a nonsporulating anaerobe suitable for use in testing the growth promoting qualities of Fluid Thioglycollate Medium. Selection was based on a comparison of the growth of these organisms in control thioglycollate with that in modified thioglycollate, i.e., media from which one ingredient had been deleted. In this study, B. vulgatus 8482 (ATCC) proved to be more fastidious in its growth requirements than the other cultures tested.

(b) Clostridium botulinum Toxin and Antitoxin: Because of the demands of other work little progress has been made on developing improved tests for botulism antitoxin. However, studies on the production and characterization of botulinum toxins are in progress.

(c) Coral Snake Venom and Antivenin: Research conducted in the Laboratory provided basic data leading to the licensing of coral snake antivenin in Spetember 1967. Through plasmapheresis of hyperimmunized goats a preparation has been obtained which is being standardized for use as a standard antiserum for coral snake antivenin. Studies of venom from various species of coral snakes indicate that venoms from a few species are not neutralized by antisera prepared from venom of other species. However, some degree of cross-neutralization occurs among the majority of species. Venoms from various species and subspecies of coral snakes have been characterized by acrylamide disc electrophoresis and agar gel electrophoresis. Preliminary work involving attempts to characterize antivenins by agar gel immuno-electrophoresis has proved promising. The work with disc electrophoresis of venoms suggests that this technique could be used in identifying species and subspecies of coral snakes.

(d) Opacity Standard: The U. S. Opacity Standard used in the standardization of cell concentrations of bacterial vaccines and of bacterial challenge suspensions used in potency tests is composed of finely divided glass particles suspended in water. During use and storage the opacity of the standard gradually lessens due to changes in particle size and adherence of particles to the walls of the container. Another undesirable feature is the difficulty of reproducing exactly the properties from lot to lot. For example the present international preparation differs from all previous international

preparations as well as from the U. S. standard. This is causing serious problems in laboratories throughout the world. It has been reported that a stable opacity preparation can be prepared by suspending finely divided titanium dioxide in a solid resin. We have initiated this project to determine if such a preparation is feasible for use as the U. S. Opacity Standard.

(e) Smallpox Vaccine: Studies associated with problems in the control of smallpox vaccine have been directed toward dried smallpox vaccine intended for administration by the jet gun. Because a portion of the vaccine is injected below the skin by the jet gun, it has been considered advisable to require that smallpox vaccine intended for jet injection be free of viable bacteria. Present vaccine prepared in the skin of calves is difficult to prepare free of viable bacteria. When present in relatively small numbers in smallpox vaccine, bacteria are difficult to detect. Therefore, studies have been initiated to devise sensitive techniques for determining the level of viable aerobic and anaerobic bacteria in smallpox vaccine. These tests will then be applied to the control testing of the vaccine.

(f) U. S. Standard Tetanus Antitoxin: The supply of the dried master used in the preparation of the U. S. Standard Antitoxin is nearly depleted. This dried preparation was adopted in 1907 and has been in use since that date. Five lots of equine tetanus antitoxin and one lot of tetanus immune globulin (Human) are being studied in order to select one having suitable properties for use as a replacement for the master preparation. Work to date indicates that avidity of these preparations can be changed depending upon the type of toxin used. In addition this study and testing performed in the Laboratory on lots submitted for release indicates that tetanus immune globulin (Human) differs from tetanus antitoxin (equine) in the nature of the toxin-antitoxin neutralization reaction which occurs. Studies are in progress to investigate these differences. One obvious difference is in the type of antigen used to produce the antibodies. Horses are hyperimmunized with relatively crude toxin following preliminary doses of toxoid. Humans are hyperimmunized with toxoid of relatively high purity.

(g) RIF-free Yellow Fever Virus Seed: In collaboration with LVR and LP the safety and antigenicity of RIF-free primary and secondary yellow fever seed virus preparations derived from the RIF-free virus prepared by LVR were studied. These preparations were found to be as safe and antigenic as the present seed by use of tests recommended by WHO and the U. S. Minimum Requirements for Yellow Fever Vaccine.

CONTRACT NARRATIVES
LABORATORY OF CONTROL ACTIVITIES
July 1, 1967 through June 30, 1968

MIDWEST RESEARCH INSTITUTE (PH43-67-679)

Title: Studies on the biologic activity of oil adjuvants

Contractor's Project Director: Dr. John O. Macfarlane

Project Officer (DBS): Dr. Pinya Cohen

Assistant Project Officer (DBS): Dr. M. Carolyn Hardegree

Objectives: Determine the safety of oil adjuvants with regard to oncogenic or other pathologic activity.

Methods Employed: Groups of inbred and random bred mice are injected subcutaneously with mineral oil, emulsifier and antigen, singly and in emulsion. Other groups receive the same treatment plus a known chemical carcinogen. Gross and microscopic pathologic examinations of the mice are made over a 1-1/2 to 2 year period to determine the development and/or acceleration of tumors or other pathologic activity.

Major Findings: Mice given two biweekly subcutaneous injections of two lots of mineral oil, two lots of Arlacel A emulsifier, mixtures of these oils and emulsifiers, and water-in-oil emulsions of influenza vaccine and house dust extract have been observed for periods of 30 to 41 weeks. A small number of tumors have developed in male swiss mice that received these materials. None of the controls have any tumors. The significance of these tumors cannot be completely determined until more time has elapsed.

In co-carcinogenesis studies Arlacel A when mixed with a known carcinogen caused tumors to appear in mice earlier than in control mice injected with carcinogen only.

Significance to DBS Program and Biomedical Research: Evaluation of adjuvant preparations in animals under a variety of conditions will provide data on the safety of these materials. The results of this and other studies will provide a basis for decisions on the use and potential safety of oil adjuvants in humans.

Proposed Course of Project: This contract will continue through February 1969. Additional studies on peanut oil adjuvants are planned.

Date Contract Initiated: February 24, 1967

Current Annual Level: $171,250.00

UNIVERSITY OF OREGON MEDICAL SCHOOL (PH43-67-675)

Title: Investigations on the mechanisms of chemical carcinogenesis

Contractor's Project Director: Dr. A. Wesley Horton

Project Officer (DBS): Dr. Pinya Cohen

Assistant Project Officer (DBS): Dr. M. Carolyn Hardegree

Objectives: Develop short-term biophysical tests that correlate with existing long-term biological test systems designed to detect chemical carcinogenic activity; apply such tests to adjuvant components and their fractions; conduct studies on the composition of mineral oil; and determine the effects of carcinogens on the immunologic response of guinea pigs and mice.

Methods Employed: Comparative biophysical measurements are taken of both standard, known chemical carcinogens and various adjuvant components and their fractions. Components and fractions which yield data similar to known carcinogens are subjected to confirmatory animal tests.

Studies on the composition of mineral oil employ high efficiency vacuum distillation and liquid thermal diffusion.

Major Findings: In a model system which simulates membrane transport the most promoted carcinogenic response is associated with chain lengths in the paraffins that produced the maximum retardation of interfacial transport. Monomolecular film studies show that sterols which are found in cell membranes interact strongly with carcinogens and not at all with noncarcinogens. Immunological work has shown that mineral oil enhances the immunoblast response to contact sensitizers in mice and that this response may vary with the composition of the mineral oil.

Significance to DBS Program and Biomedical Research: These studies may accelerate the development of quick, *in vitro* tests to determine the presence of carcinogens in components used to prepare adjuvants; elucidate mechanisms of action of carcinogens at the cellular and subcellular level; and establish criteria for the safety of adjuvant preparations.

Proposed Course of Project: The contract has been renewed until March 31, 1969.

Date Contract Initiated: March 17, 1967

Current Annual Level: $67,990.00

CONTRACT NARRATIVES
LABORATORY OF CONTROL ACTIVITIES
July 1, 1967 through June 30, 1968

SOUTHWEST RESEARCH INSTITUTE (PH43-67-1104)

Title: The metabolic fate of an emulsion containing mineral oil and Arlacel A using carbon - 14 tracers.

Contractor's Project Director: Dr. James N. Bollinger

Project Officer (DBS): Dr. Pinya Cohen

Assistant Project Officer (DBS): Dr. M. Carolyn Hardegree

Objectives: Determine the metabolic fate, distribution and any adverse cellular effects produced in the animal body by an emulsion containing Drakeol 6VR mineral oil and Arlacel A emulsifier.

Methods Employed: Emulsions of radiolabeled mineral oil and emulsifier are injected subcutaneously and intramuscularly into rats and squirrel monkeys. The animals are placed in metabolism cages and quantitative measurements are made of the urine, feces and respiratory CO_2 collected. At various intervals animals are killed and the distribution of radioactivity in the major organs and biochemical classes (proteins, lipids, etc.) is determined. Gross and microscopic pathologic examinations are made.

Major Findings: Preliminary data indicate that mineral oil does not readily leave the site of injection even after two months. It appears that the oil can be metabolized in the liver as it leaves the site of injection, but that it may accumulate in some organs unmetabolized.

Significance to DBS Program and Biomedical Research: The study may provide an approach for identifying the specific sites in the animal body which may be affected upon the introduction of an oil emulsion. This information will be useful in assessing the safety of oil adjuvant preparations in humans.

Proposed Course of Project: Contract will terminate June 30, 1968.

Date Contract Initiated: May 12, 1967

Current Annual Level: $40,636.00

I.I.T. RESEARCH INSTITUTE (PH43-67-1101)

Title: Chemical and Physicial Analysis of Oils and Emulsifiers

Contractor's Project Director: Dr. H. J. O'Neill

Project Officer (DBS): Dr. Pinya Cohen

Assistant Project Officer (DBS): Dr. M. Carolyn Hardegree

Objectives: Isolate and identify the major and minor components in Drakeol 6VR mineral oil and Arlacel A emulsifier.

Methods Employed: Detailed compositional analyses are performed through the combined use of column, thin-layer and gas chromatography; nuclear magnetic resonance; and infrared and mass spectrometry. Characterization of mineral oil will provide information on the ratio and structure of paraffins, isoparaffins and cycloparaffins over the entire range of molecular weights and on the presence of other nonparaffinic hydrocarbons. Analyses of Arlacel A include studies on the purity and homogeneity of the fatty acid and carbohydrate components.

Major Findings: Ultraviolet spectra of mineral oil indicates presence of aromatics resembling phenolic compounds. Gas chromatographic and ultraviolet analyses of Arlacel A showed presence of cyclic components which form thermal cyclization of unsaturated fatty acids. These compounds are known to be toxic when placed in the diets of weanling rats. Synthetic procedures to prepare a pure mannide monooleate (Arlacel A) have been initiated. Qualitative and quantitative analyses of mineral oil and Arlacel A imulsifier are in progress.

Significance to DBS Program and Biomedical Research: The procedures developed and analytical findings will be used to develop new criteria and specifications for defining the composition and safety of light mineral oil and mannide monooleate emulsifier. Particular emphasis will be placed on the identification of carcinogens, potential carcinogens, and other harmful agents.

Proposed Course of Project: This contract will terminate June 22, 1968, however, additional analyses on peanut oil adjuvant are tentatively planned.

Date Contract Initiated: May 23, 1967

Current Annual Level: $42,370.00

Serial No. DBS 143
1. Control Activities
2. Reference Standards
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Bacteroides vulgatus for Testing Fluid Thioglycollate Medium.

Previous Serial Number: None

Principal Investigator: Jane F. Farber

Other Investigator: Edward B. Seligmann Jr., Ph.D.

Cooperating Units: None

Man Years
Total: 0.25
Professional: 0.25
Other: 0

Project Description:

Objectives: To select a non-sporeforming anaerobe suitable for testing the growth promoting properties of fluid thioglycollate medium as used in sterility testing.

Methods Employed: Various anaerobic bacteria were tested for their ability to initiate growth from small innocula in fluid thioglcyollate medium and in variations of this medium from which one ingredient at a time had been deleted. The organism most adversely effected by the modifications was considered to be the best test organism.

Major Findings: The growth requirements of Bacteroides ovatus and B. vulgatus were compared with those of Clostridium tetani and Clostridium chauvoei in order to select a nonsporulating anaerobe suitable for use in testing the growth promoting qualities of Fluid Thioglycollate Medium. Selection was based on a comparison of the growth of these organisms in control thioglycollate with that in modified thioglycollate, i.e., media from which one ingredient had been deleted. In this study, B. vulgatus 8482 (ATCC) proved to be more fastidious in its growth requirements than the other cultures tested.

Significance to Bio-Medical Research and the Program of the Division: In order to demonstrate the validity of each sterility test the growth promoting properties of the medium must be demonstrated. Manufacturers lacking facilities for handling spore-forming microorganisms are unable to use

spore-forming anaerobes usually used in such testing. A non-sporeforming, fastidious anaerobe would provide such manufacturers with a satisfactory test organism. A strain of Bacteroides vulgatus was found to fulfill these requirements.

Proposed Course of Project: Completed

Honors & Awards: None

Publications: Farber, J.F. and Seligmann Jr., E.B. Bacteroides vulgatus 8482 (ATCC): A Nonspore-Forming Anaerobe for Testing Fluid Thioglycollate Medium. Bacteriological Proceedings 1968, Abstracts, 28, 1968, presented at 68th Annual Meeting ASM, 1968.

Serial No. DBS 144
1. Control Activities
2. Reference Standards
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Clostridium botulinum Toxin and Antitoxin

Previous Serial Number: None

Principal Investigator: Lee J. Stevan, M.S.

Other Investigators: Edward B. Seligmann Jr., Ph.D.

Cooperating Units: None

Man Years
Total: 0.25
Professional: 0.25
Other: 0

Project Description:

Objectives: To establish the relationship of various nutritional components of the growth medium to the biochemical structure of toxins of various strains of Clostridium botulinum types A, B, C, and E.

Methods Employed: Techniques of immunochemistry, fractionation procedures, biochemistry and bacteriology.

Major Findings: Studies were started on the production and characterization of bolulinum toxins.

Significance to Bio-Medical Research and the Program of the Division: Potency testing of botulism antitoxins has been unsatisfactory primarily due to inadequate test toxins. A better understanding of the nature of botulinum toxins will lead to more satisfactory test procedures and possibly a better understanding of the mechanism of action of both the toxin and the antitoxin.

Proposed Course of Action: The work has just been initiated. It will be expanded considerably during the coming year.

Honors and Awards: None

Publications: None

Serial No. DBS 145
1. Control Activities
2. Reference Standards
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Coral Snake Venom and Antivenin

Previous Serial Number: None

Principal Investigator: Pinya Cohen, Ph.D.

Other Investigators: Edward B. Seligmann Jr., Ph.D.
John H. Dawson
Lee J. Stevan, M.S.
William H. Berkeley

Cooperating Units: None

Man Years
Total: 2.1
Professional: 1.6
Other: 0.5

Project Description:

Objectives: Establishment of standards for coral snake antivenin. Study of the immunogenic properties of coral snake venoms from various species of snakes. Electrophoretic studies of coral snake venoms.

Methods Employed: Hyperimmunization of various species of animals. Venom neutralization tests. Immunoelectrophoresis, agar gel diffusion, acrylamide disc electrophoresis and agar gel electrophoresis.

Major Findings: Antisera have been prepared in rabbits, goats, ponies and burros against venom from a number of species of coral snake. Cross-neutralization tests indicate that two of these species have venoms which cannot be neutralized by anything other than homologous antiserum. Electrophoretic patterns indicate that purity and species identity of coral snake venoms may be determined by use of these techniques.

Significance to DBS Program and Bio-Medical Research: As an outcome of work performed in the Laboratory Wyeth Laboratories was licensed in September 1967 for Antivenin (Micrurus fulvius) which is effective against the venom of all coral snakes found in the United States except for Micruroides euryxanthus, the Arizona coral snake. Studies

on cross-neutralization of venom from various species of coral snake are leading to an understanding of the extent of usefulness of coral snake antivenins for treatment of bites from coral snakes found throughout North, Central and South America.

Proposed Course of Project: Completion of work in progress on cross-neutralization and immunochemical properties of venoms from various species. Completion of work in progress on the *in vivo* effectiveness of antivenins in treating envenomation.

Honors and Awards: None

Publications: Cohen, P., Dawson, J. H. and Seligmann Jr., E.B. Cross-Neutralization of *Micrurus fulvius* (Coral Snake) Venom by Anti-*Micrurus carinicanda dumerilii* Serum. *The American Journal of Tropical Medicine and Hygiene*. In press for Spring 1968.

Serial No. DBS 146
1. Control Activities
2. Reference Standards
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Standardization of Reference Diphtheria Antitoxin For Flocculation Test

Previous Serial Number: None

Principal Investigator: Jane F. Farber

Other Investigator: Edward B. Seligmann Jr., Ph.D.

Cooperating Units: None

Man Years
Total: 0.25
Professional: 0.25
Other: 0

Project Description:

Objectives: To select a suitable diphtheria antitoxin to replace the present Reference Diphtheria Antitoxin for Flocculation Test upon depletion and to establish Lf and L+ values equivalent to the present reference.

Methods Employed: Candidate antitoxins and the reference antitoxin were flocculated against the control toxin by both the Ramon and Dean - Webb methods to determine the Lf unitage and the Kf value. The L+ unitage of the antitoxins were established by guinea pig neutralization tests.

Major Findings: A diphtheria antitoxin that possessed suitable characteristics was selected from four candidate sera. The Lf unitage was established based on comparison tests with the reference antitoxin. A Kf value similar to that of the reference was one of the criteria used for selection. By comparing the neutralizing capacity of the candidate antitoxin to that of the U.S. Standard Diphtheria Antitoxin an L+ value was established.

Significance to Bio-Medical Research and the Program of the Division: Reference Diphtheria Antitoxin for Flocculation Test provides the division, biologic manufacturers and research workers in the field, with a rapid means of measuring the activity of diphtheria antitoxins, toxins and toxoids in the form of final products and also during the manufacturing process.

Proposed Course of Project: Following the vialing and drying of the antitoxin the Lf and L+ values will be reconfirmed by the flocculation and animal neutralization tests. This preparation will then be ready to be presented to the manufacturers and other interested workers for their review as a candidate reference. If found to be satisfactory, it will be adopted as the new reference.

Honors and Awards: None

Publications: None

Serial No. DBS 147
1. Control Activities
2. Reference Standards
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Development of an Improved Opacity Standard

Previous Serial Number: None

Principal Investigators: Jane F. Farber

Other Investigators: Edward B. Seligmann Jr., Ph.D.

Cooperating Units: None

Man Years

Total: 0.25
Professional: 0.25
Others: 0

Project Description:

Objectives: 1) To prepare a stable solid opacity standard by suspending finely divided particles in a resin 2) to standardize this preparation in terms of the current opacity standard which is an aqueous suspension of glass particles 3) to determine the practicality of distributing a solid standard for use by manufacturers.

Methods Employed: A method will be developed whereby varying concentrations of titanium dioxide can be evenly suspended in viscous melted resin which will then be allowed to harden. The concentration having the same light transmission as the U.S. Opacity Standard will be selected. The design of instruments used in measuring percent of light transmission will be considered in determining the feasibility of distributing the solid opacity standard in suitably sized tubes.

Major Finding: Work on this project has just been initiated.

Significance to Bio-medical Research and the Program of the Division: The U.S. Opacity Standard is used to standardize the cell concentrations of bacterial vaccines and to achieve lot to lot consistency. The bacterial cell concentration of challenge doses in potency tests are also adjusted with this standard. Because of the relative instability of the current aqueous suspension of glass particles a new lot must be prepared and distributed every few years. It is extremely difficult to reproduce the

exact properties of a suspension of finely divided glass particles. Differences among lots and changes in a lot during use can lead to variation among lots of vaccine. As an example of the problems created by different lots of suspended glass opacity preparations the current lot of international reference differs significantly from any previous U.S. or international preparation and laboratories throughout the world are having difficulties in using it in the preparation of vaccines. Because of its unique characteristics a permanent titanium dioxide-resin type of standard would resolve many of these recurring problems.

Proposed Course of Project: To prepare test batches of opacity material using different resins and titanium dioxides to determine the most suitable combination for use as the U.S. Opacity Standard. A sufficiently large quantity of this material will then be prepared and tubed.

Honors and Awards: None

Publications: None

Serial No. DBS 148
1. Control Activities
2. Control Tests
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Development of Sterility Test Procedures for Testing Dried Smallpox Vaccine For Jet Gun Injection

Previous Serial Number: None

Principal Investigators: Vernon J. Fuller, Ph.D.
Robert W. Kolb, M.S.

Other Investigator: Ruben Marquina

Man Years
Total: 0.10
Professional: 0.05
Other: 0.05

Project Description:

Objectives: To determine the incidence of contamination of commercial lots of dried smallpox vaccine for jet gun injection.

Methods Employed: Preliminary sterility testing with varying doses was carried out by the pour plate and test tube methods. The tube method was selected as the method of choice since the rate of contamination obtained by either method was essentially the same and because tests could be carried out more expeditiously. The lower limit of sensitivity of the test method was determined to be 10 doses of vaccine.

Major Findings: Of the 20 lots of vaccine tested, two lots had a high incidence of contamination, that is, each lot had one or more positive culture tube per each of 300 doses tested. Seventeen lots had a low incidence of contamination and one lot was negative for the presence of bacteria.

These initial findings indicate that the test method is sufficiently sensitive to detect minimal contamination of dried smallpox vaccine for jet gun injection.

Significance to Bio-Medical Research and the Program of the Division: The data collected in this and additional studies will serve as a basis for drafting sterility standards for dried smallpox vaccine for jet gun injection.

Proposed Course of Project: Experiments will continue in the development of a suitable sterility test procedure.

Honors & Awards: None

Publications: None

Serial No. DBS 149
1. Control Activities
2. Control Tests
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Comparison of Titrations on the Chorioallantoic Membrane of Chick Embryos with the Rabbit Scarification Technique for the Potency Assay of Smallpox Vaccine.

Previous Serial Number: None

Principal Investigators: Vernon J. Fuller, Ph.D.
Robert W. Kolb, M.S.

Other Investigators: Ruben Marquina
Harry Aylor

Man Years: Total: 1.7
Professional: 0.8
Other: 0.9

Project Description:

Objectives: To determine the titer of the U.S. Reference in embryonated chicken eggs, to compare the potency of the U.S. Reference Smallpox Vaccine with that of the International Reference Smallpox Vaccine by the CAM and RS test methods, and to compare the potency of commercial smallpox vaccine in relation to that of the U.S. Reference by the CAM and RS test methods.

Methods Employed: The chorioallantoic membrane technique and the official U.S. rabbit scarification method were used in this study.

Major Findings: A single test performed on each of 107 ampoules of the U.S. Reference gave a mean titer of $10^{8.1}$ PFU/ml with a standard deviation of ± 0.2 log.

The mean value obtained for three ampoules of the International Reference was $10^{7.8}$ PFU/ml.

Eighty-nine percent of the lots of the commercial smallpox vaccine tested by the CAM and RS methods demonstrated potencies comparable to the U.S. Reference.

Significance to Bio-Medical Research and the Progress of the Division: The data obtained in this study served as the basis for the drafting of alternate potency standards for smallpox vaccines.

Proposed Course of Project: This study has terminated and the results have been published in a scientific journal.

Honors and Awards: None

Publication: Fuller, V.J., and Kolb, R.W. Comparison of Titrations on the Chorioallantoic Membrane of Chick Embryos with the Rabbit Scarification Technique for the Potency Assay of Smallpox Vaccines. Applied Microbiology: 16: 458-462, 1968.

Serial No. DBS 150
1. Control Activities
2. Reference Standards
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Replacement of the U. S. Standard Tetanus Antitoxin

Previous Serial Number: None

Principal Investigators: Edward B. Seligmann Jr., Ph.D.
Jane F. Farber

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 0.5
Professional: 0.5
Other: 0

Project Description:

Objectives: To select a new lot of antitoxin that will prove suitable for use as the master dried U. S. Standard Tetanus Antitoxin, the supply of which is nearly depleted.

Methods Employed: Protective unitage is determined by comparing the toxin neutralizing capacity of the candidate antitoxins with that of the standard antitoxin in mice and guinea pigs. A comparison of these L+ values obtained at the L+/10, L+/100 and L+/1000 test levels indicates the degree of avidity of each antitoxin. As an indication of the stability of the toxin-antitoxin mixtures during the time necessary to conduct a test, the LD_{50} of the toxin and the L+ value of the antitoxin were determined after 1 hr. and up to 48 hrs. incubation periods. Periodically, neutralization tests are performed on glycerinated candidate antitoxins that are stored at 37°C. to demonstrate stability in an excellerated storage stability test. The Lf values of the antitoxins are determined by the Ramon flocculation tests.

Major Findings: (1) Stability of glycerinated antitoxin stored at 37°C.: Lots C and F - L+ values have decreased about 40% after 20 months; Lot E - L+ value has decreased about 20% after 8 months.

(2) Avidity
A. The L+ values of all the antitoxins when combined with tetanus toxin Lot 0 (five equine and one tetanus immune globulin) increased with the

dilution factor of the test (L+/100 L+/1000). The values of these same antitoxins when combined with a different tetanus toxin, Lot T-1, decreased with the dilution factor of the tests (L+/1000 L+/100). In the few tests performed with a more recently acquired third toxin, Lot TD, the L+ values of the antitoxin also decreased with the dilution of the test (L+/1000 L+/100).

B. Forty-eight hour stability of toxin-antitoxin mixtures: On combining the six lots of antitoxin with either Toxins Lot 0 or Lot T-1 the L+ values increased after an incubation period of 48 hours. One test performed with Toxin Lot TD produced similar results. A few tests with tetanus immune globulin antitoxin also yielded higher values after 48 hours but they were of a lesser magnitude. In all tests the LD_{50} value of the toxin remained stable.

Significance to Bio-Medical Research and the Program of the Division: This study will aid in the selection of an antitoxin possessing the most suitable characteristics for use as the official U. S. Standard Tetanus Antitoxin. Resolution of the problems encountered will further clarify the mechanism of action involved in the animal neutralization test which is the official potency for tetanus antitoxin. Problems unique to the testing of tetanus immune globulin (Human) have been encountered and are being studied.

Proposed Course of Project: Work is underway to prepare a rabbit antitoxin with Toxin Lot 0. Thus far, this study which has been conducted with various combinations of toxins and antitoxins has failed to resolve problems encountered in testing the candidate antitoxins. Repetition of these experiments with a homologous toxin and antitoxin may clarify these problems and thereby assist in selecting the best suited antitoxin for use as the official standard.

Honors & Awards: None

Publications: None

Serial No. DBS 151
1. Control Activities
2. Control Tests
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Suppression of Tuberculin Skin Reactions

Previous Serial Number: None

Principal Investigators: Robert W. Kolb, M.S.
Harold Baer, Ph.D.

Other Investigators: None

Man Years:
Total: 0.25
Professional: 0.25
Other: 0

Project Description:

Objectives: To determine the effect of various strengths of tuberculin on skin reactions when tested simultaneously on the same animal.

Major findings: Studies on the suppression of tuberculin reactions induced by the injection of high concentrations of tuberculins into sensitized guinea pigs were completed during the year. The results of the study was published in a scientific journal.

Proposed Course of Project: Completed

Honors and Awards: None

Publications: Baer, Harold and Kolb, Robert W. Suppression of the tuberculin reaction by high concentrations of tuberculin and the relationship of this phenomenon to the potency assay of tuberculin. Jour. Immun. 98: 1015 - 1019, 1967.

Serial No. DBS 152
1. Control Activities
2. Control Tests
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1 1967 through June 30 1968

Project Title: Statistical Evaluation of the Guinea Pig Potency Assay and Standardization of Old and PPD Tuberculins.

Previous Serial Number: None

Principal Investigators: Robert W. Kolb, M.S.
Harold Baer, Ph.D.
Clifford J. Maloney, Ph.D.

Other Investigators: None

Man Years:
Total: 0.25
Professional: 0.25
Other: 0

Project Description:

Objectives: To present a statistical analysis of potency test procedures used in the control testing and standardization of U. S. licensed tuberculin products.

Methods Employed: Tuberculins were standardized by comparison with U. S. Standard Tuberculin OT or PPD in guinea pigs sensitized with killed Mycobacterium tuberculosis suspended in oil. Potency data collected over approximately a five year period are being analyzed by various statistical procedures by the DBS Biometrics Section.

Major Findings: The analysis of data collected over a two and a half year period showed that by the methods used dose response curves were linear and parallel and that relative potencies could be obtained within an error of less than 20%.

Significance to Bio-Medical Research and the Progress of the Division: The data obtained in this study will serve as a basis for the revision of official laboratory potency standards for tuberculins.

Proposed Course of Project: To continue the analyses and prepare a summary report for publication in a scientific journal.

Honors and Awards: None

Publications: None

Serial No. DBS 153
1. Control Activities
2. Reference Standards
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Development of a Reference Typhus Vaccine and an Improved Potency Test

Previous Serial Number: None

Principal Investigator: Edward B. Seligmann Jr., Ph.D.

Other Investigators: Robert W. Kolb, M.S.
Otis L. Green
Harry T. Aylor
Elmer C. Russell

Cooperating Units: None

Man Years
Total: 0.1
Professional: 0.03
Other: 0.07

Project Description:

Objectives: Establishment of a suitable Reference Typhus Vaccine preparation. Development of an imporved potency test for typhus vaccine.

Methods Employed: Standard potency test described in the Minimum Requirements for Typhus Vaccine and a variety of modifications including antigen extinction type tests.

Major Findings: This project was started late in the year. Preliminary results indicate that a single dose antigen extinction assay is feasible.

Significance to Bio-Medical Research and the Program of the Division: The effectiveness of present typhus vaccine has been questioned by the scientific community concerned with the use of typhus vaccine in the armed services. A reference vaccine would aid in assuring more consistency among production lots. In addition a more sensitive potency test is required in order to detect differences among lots more reliably. The present potency test consists of two immunizing doses which tends to mask differences in vaccine activity.

Proposed Course of Project: Continuation of studies.

Honors & Awards: None

Publications: None

Serial No. DBS 154
1. Control Activities
2. Reference Standards
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Bactericidal Activity of Wasp Extracts

Previous Serial Number: None

Principal Investigators: Edward B. Seligmann Jr., Ph.D.
Jane F. Farber

Other Investigators: Dr. Allen W. Benton, Department of Entomology, Pennsylvania State University

Cooperating Units: Department of Entomology, Pennsylvania State University

Man Years

Total: 0.2
Professional: 0.1
Other: 0.1

Project Description:

Objectives: 1) To detect the presence of and evaluate the factors found in wasps and hornets which inhibit growth of bacteria and fungi 2) to isolate and identify the inhibiting agent(s).

Methods Employed: Extracts prepared from wasps and sterilized by filtration are tested for inhibitory effects on the growth of a variety of microorganisms.

Major Findings: Initial tests have demonstrated an inhibitory factor in bodies of White Faced Hornet Queens upon the growth of several gram negative bacteria and spore-forming anaerobes.

Significance to Bio-medical Research and the Program of the Division: This project originated out of an observation that bodies of wasp and hornet queens failed to deteriorate in a freezer after a power failure. The bodies of other insects stored in the same freezer decomposed. The queens normally overwinter either in the ground or in suspended nests. It is reasonable to assume that they may be resistant to invasion by soil microorganisms. Substances inhibitory to certain classes of bacteria found in the soil and to certain fungi could be of significance in the treatment of diseases and injuries of man.

Proposed Course of Project: 1) The inhibitory factor(s) will be tested against a variety of bacteria and fungi 2) an attempt will be made to isolate and identify the inhibitory agent(s).

Honors and Awards: None

Publications: None

Serial No. DBS 155
1. Control Activities
2. Reference Standards
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: RIF - free Yellow Fever Virus Seed

Previous Serial Number: None

Principal Investigator: Jane F. Farber

Other Investigator: Edward B. Seligmann, Jr., Ph.D.

Cooperating Units: Laboratory of Virology & Rickettsiology
Laboratory of Pathology

Man Years
Total: 0.75
Professional: 0.25
Other: 0.5

Project Description:

Objectives: To compare the safety and antigenicity of the RIF-free primary and secondary yellow fever seed viruses to that of the current primary and secondary seed viruses.

Methods Employed: The characteristics of the seed viruses were compared by the methods described in WHO Technical Report Series No. 179, 1959 whereby a group of monkeys were immunized with the viruses. The level of viscerotropism of each virus was determined by mouse titration of the circulating virus present in the monkey blood on specific post immunization days. Within 30 days after immunization, the antibody level of the pre and post monkey sera was demonstrated by neutralization of a challenge virus in mice. The appearance of clinical manifestations of encephalitis in monkeys indicated the degree of neurotropism of the virus.

Major Findings: No significant differences in safety or antigenicity were detected between the RIF-free seed virus preparations and the present seed virus lots. In this study it was demonstrated that the monkey serum antibodies were more effective in neutralizing a homologous virus strain than a heterologous strain.

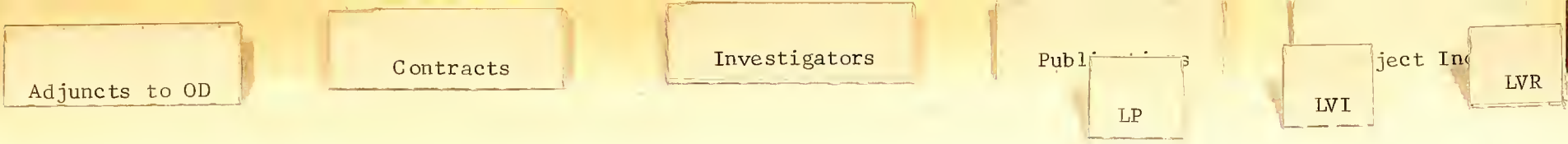
Significance to Bio-Medical Research and the Program of the Division: Before use of a RIF-free yellow fever seed virus in the production of vaccine it was essential to demonstrate that manipulation of the virus

necessary to free it of RIF had not altered its characteristics of safety and antigenicity.

Proposed Course of Project: The findings will be prepared for publication in conjunction with a description of the method used to remove RIF.

Honors & Awards: None

Publications: None


Adjuncts to OD
Contracts
Investigators
LP
LVI
LVR

Annual Report of the Laboratory of Pathology
Division of Biologics Standards
July 1, 1967 through June 30, 1968

The Laboratory of Pathology has a continuing program to study the pathogenesis of infectious diseases using animal models. This program is designed to meet the overall needs of the Division in regard to the safety and efficacy of new biological products and also those which have been licensed for many years. In the past, many of the activities of the laboratory have centered on the testing of the neurovirulence properties of strains of poliovirus in order to develop the most sensitive tests for the safety of the inactivated and live attenuated vaccines. In addition, since large numbers of commercial lots of Poliovirus Vaccine, Live, Oral, had been submitted for release, much of the efforts of the laboratory were directed toward the neurovirulence testing of these lots. This year, as last, with the extremely low level of poliomyelitis in the United States and in the world, the number of lots of vaccine submitted for testing was very small. However, the laboratory does perform neurovirulence tests on all of these lots at the present time and, in addition, continues to participate in developmental studies aimed at standardizing testing of vaccines throughout the world. (Project Number 41, p. 132)

Much of the effort of the laboratory is now being directed toward research in, and the development of, significant test systems for the newer attenuated viral vaccines. Thus, programs are under way to study the pathologic alterations induced by wild adenoviruses, rubella virus, and mumps virus in experimental animals so as to meaningfully evaluate the attenuation of such viruses in live virus vaccines. These studies are being done in collaboration with other laboratories within the Division.

In collaboration with the Laboratory of Viral Immunology, studies have continued of wild and attenuated rubella virus both in pregnant and nonpregnant rhesus monkeys. The virus can infect these animals and multiply, although no clinical syndrome develops in either the adult or the fetus. (Project Number 112, p. 143)

In collaboration with the Laboratory of Virology and Rickettsiology and Dr. Kendall Smith, studies of wild and "vaccine" strains of human adenovirus 4 are in progress. Attempts to infect monkeys appear to be unsuccessful but the virus apparently does cause an inflammatory reaction when placed directly within the central nervous system. This reaction can be found whether crude or purified virus is used. This phenomenon is of great interest and further studies are being undertaken to elucidate the mechanism. (Project Number 112, p. 143)

A similar reaction has been found to occur when mumps virus (wild or attenuated strains) is inoculated into the central nervous system of monkeys. However, the virus does not replicate within the CNS and this reaction does not appear to be related to neurotropism in man. Furthermore, the attenuated (vaccine) strain of mumps virus will not infect monkeys when

inoculated parenterally, although an antibody response is obtained. These studies resulted in the promulgation of Additional Standards for Mumps Virus Vaccine, Live, with the subsequent issuance of a license for this product. (Project Number 112, p. 143)

Yellow Fever Vaccine (17D strain) has also been under study in collaboration with the Laboratory of Virology and Rickettsiology and the Laboratory of Control Activities. The neurovirulent properties of this virus have been found to be stable even when a virus of the avian leucosis complex has been removed from the seed virus pool. Since Yellow Fever is a disease that affects the central nervous system as well as the viscera, and since the vaccine has neurotropic properties for rhesus monkeys, control of the stability of this neutropism at a low level is essential for use of this new and more purified vaccine in man. The immunogenicity of this derivative of the primary seed strain and its stability are being investigated. (Project Number 112, p. 143)

The Section of Histopathology has expanded its activities in electron microscopy greatly and has developed excellent techniques. Studies of the ultrastructure of the central nervous system are designed to complement previous similar studies using light microscopy. (Project Number 45, p. 137) As the Section perfects its techniques for ultrastructural studies, it is expected that collaborative programs using such techniques will increase and expand. The Section of Histopathology has continued to perform clinical laboratory techniques and has acquired and learned to operate an automatic counter for assaying red and white blood cells. This has enabled the technical staff to perform such procedures with great efficiency.

Dr. Clark has expanded his studies of myelinization of the central nervous system of animals to include studies of demyelinating diseases, such as experimental allergic encephalomyelitis, and has initiated a study of congenital deformities of the central nervous system of animals. Such a baseline study is essential to subsequent evaluation of teratogenesis in relationship to the use of biological products.

The Section of Animal Testing, in addition to performing neurovirulence tests on all lots of Poliovirus Vaccine, Live, Oral, and selected lots of Poliomyelitis Vaccine, Measles Vaccine, Live, and Mumps Vaccine, Live, has recently established a colony of South American monkeys, namely, marmosets (Saguinus mystax). Interest in this primate has increased greatly recently since it has been found to be susceptible to Rous sarcoma virus and has been thought possibly to be of use in the study of the pathogenesis of infectious hepatitis. Therefore, in October, 1967, the Animal Test Section obtained and established a colony of 85 marmosets. The purpose of establishing the colony now was to develop skills in the handling, care, and breeding of these animals as well as to develop background information concerning the microorganisms harbored by them. Only when these goals have been accomplished, can these animals be considered suitable for experimental studies. The colony is under the direct supervision of a research microbiologist who is a new addition to the staff of the Laboratory and who has established the necessary laboratory facilities to perform such studies. The colony has

decreased from 85 to 69 in the six months since it has been established, the primary cause of death being the overwhelming effects of parasitic infestation. The Laboratory has been very fortunate in being able to consult with the veterinarians in charge of the Primate Quarantine Unit, Laboratory Aids Branch, as to the medical care of the marmosets. This has been most helpful since the Laboratory lost its own veterinarian in October, 1967. It is hoped that, by the time the colony of marmosets is a year old, a complete microbiological profile will be established, methods of handling will be improved, the diet will be stabilized so that weight gain will be adequate, and a nucleus of laboratory-bred marmosets will be available for further studies. (Project Number 133, p. 150)

The Chief of the Laboratory has directed most of her research efforts in the general field of the pathogenesis of infectious diseases with particular emphasis on oncogenic viruses. The pathogenesis of the particular tumors induced, the effect of altered immunologic mechanisms, and the interaction of these viruses on each other are all being studied. Attempts to prevent tumors by specialized immunization procedures are in progress. (Project Number 44, p. 134)

In addition, the Chief of the Laboratory plans, coordinates, and supervises all the collaborative research projects in which the staff of the Laboratory participate. Some of these projects are related to control activities and others to research carried out in other laboratories and institutes. Two members of the staff of the Contracts Operations office are engaged in research projects under the supervision of and in collaboration with the Chief of the Laboratory of Pathology. One, Dr. Stanley Singer, is investigating the effects of oncogenic and non-oncogenic human adenoviruses on cell cultures in attempts to find chemicals which affect the oncogenicity of these viruses. The other, Mr. Edward Fitzgerald, is studying certain hamster tumors as described under Collaborative Activities.

Collaborative (Contracts) Activities:

Dr. Amos E. Palmer of the Laboratory of Pathology was Project Officer for a contract set up to study the possible oncogenesis of biological products in newborn hamsters. Since Dr. Palmer's departure, Dr. Stanley Singer, Mr. Edward Fitzgerald, and Mrs. Claire Cox have monitored this contract closely. The activities involving this contract have been closely supervised by the Chief of the Laboratory of Pathology, who has reviewed the histologic material evolving from this contract. In addition, Mr. Fitzgerald, under the supervision of the Chief, Laboratory of Pathology, has been studying the relationship of the tumors that have occurred to the biological products used. Mrs. Claire Cox is preparing to perform certain developmental studies to further these efforts and to elucidate certain problems that have arisen.

Since many of the contracts which are under way have aspects relating to pathogenesis, the Chief of the Laboratory of Pathology works very closely with the Contracts Operations Staff, and spends much time helping to plan and execute and to review progress reports of those contracts already in

existence. The Laboratory is cooperating in performing the pathologic examinations that are necessary for certain contract operations in which the contractor cannot do these examinations himself.

Control Activities:

During the past year, the Laboratory has performed neurovirulence tests on 20 lots of Poliovirus Vaccine, Live, Oral, for release as well as tested the Reference Vaccine on 8 occasions. Three lots of Poliomyelitis Vaccine and 11 lots of Measles Virus Vaccine have been tested. Nine lots of Mumps Virus Vaccine have been tested and this vaccine was licensed for use. A total of 1485 monkeys have been used for this purpose. The safety of these vaccines has been well controlled.

In addition, the Section on Animal Testing performed 10 potency tests for Poliomyelitis Vaccine using 120 monkeys. One hundred and seventy rhesus monkeys and 100 vervet monkeys were sacrificed in order to remove kidneys for the Tissue Culture Section, LVR. Three hundred and six rabbits were similarly sacrificed.

The Histopathology Section produced 20,000 sections for light microscopy, 1400 sections for electron microscopic study, and performed 1100 complete blood counts.

The Chief of the Laboratory has served as Chairman of five license application review committees and on three other such committees. Each staff member has participated in the annual inspection program performing inspections at seven establishments.

BIONETICS RESEARCH LABORATORIES (PH43-67-1483)

Title: Studies of the Oncogenic Activity of Human Biological Products in Syrian Golden Hamsters (Mesocricetus auratus)

Contractor's Project Director: Dr. John Landon

Project Officer (DBS): Dr. Stanley H. Singer

Objectives: To study human biological products for oncogenic activity when inoculated into newborn Syrian golden hamsters.

Methods Employed: The products to be studied are purchased from commercial sources in their final containers. These compounds are coded by DBS staff members and delivered to the contractor where they are inoculated into newborn hamsters within 24 hours after birth by a single subcutaneous inoculation. These animals are observed daily, weaned at 21 days, divided into groups of 8 or less animals and observed closely for the development of palpable masses or other indications of neoplastic disease. All animals which die are subjected to thorough histopathologic examination except when cannibalism or autolysis will not permit. Transplants to weanling hamsters are attempted on all test animals which develop palpable masses. Blood serum and portions of the neoplasm are retained at -70°C. for further studies. Positive and negative controls are included in the study.

Major Findings: Animals inoculated with positive controls have developed neoplasms with high incidence and with proper latent periods. There has been a low incidence of tumor formation scattered amongst the remaining groups which is thought to represent spontaneous tumor formation in the colony.

Significance to DBS Program and Bio-Medical Research: This is one approach to determine the safety of available human biological products. Although oncogenic activity in hamsters cannot presently be related to humans, results from hamster studies should shed some light on safety of these products.

Proposed Course of Project: This project is scheduled for termination in fiscal year 1970.

Date Contract Initiated: July 1, 1966.

Current Annual Level: $303,260

Serial No. DBS 41
1. Pathology
2. Animal Testing and Histopathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Developmental Studies of Neurovirulence Testing on Poliovirus Vaccines in Monkeys

Previous Serial Number: 41

Principal Investigator: Ruth L. Kirschstein, M.D.

Other Investigators: Ronald G. Clark, Ph.D., LP-DBS
George Rusten, LP-DBS
Amos E. Palmer, D.V.M., LP-DBS

Cooperating Units: Serology Section, LVI, screens monkey serums for freedom from antibody to poliovirus.

Man Years:
Total: 5.0
Professional: 0.4
Other: 4.6

Project Description:

Objectives: To develop information concerning the reproducibility and sensitivity of neurovirulence tests of Live Poliovirus Vaccines and to refine techniques used in the test to assure safety of the vaccine.

Methods Employed: Monkeys were inoculated with vaccine or dilutions thereof either intrathalamically or intraspinally and observed for evidence of developing paralysis over a period of 17-18 days. The monkeys were then sacrificed and the central nervous system examined histologically for evidence of poliomyelitis. In addition, tissues were saved for virus isolation attempts. These studies were performed as outlined in the PHS Regulations for Poliovirus Vaccine, Live, Oral.

In addition, studies of the sensitivity of finite neuroanatomical structures to virulent and attenuated polioviruses are in progress.

Major Findings: Five Type I or Type III coded vaccines were tested repeatedly both in the Laboratory and in two European control laboratories. The results completed have been analyzed and indicate that increased neurovirulence can be found by either intraspinal inoculation of cynomolgus monkeys or intrathalamic inoculation of rhesus monkeys. The latter method, when performed correctly, better discriminates levels of neurovirulence. Differential sensitivity of finite areas of the thalamus to one strain of virulent poliovirus has been demonstrated.

Significance to Bio-Medical Research and the Program of the Division: These studies are designed to assure standardization of neurovirulence testing of poliovirus vaccines throughout the world, to assure the safety of such vaccines, and to improve testing procedures.

Proposed Course of Project: It is anticipated that collaborative studies to standardize safety testing of vaccines will be a continuing project.

Honors and Awards: None

Publications: None

Serial No. DBS 44
1. Pathology
2. Animal Testing and Histopathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Pathogenesis of Infectious Diseases

Previous Serial Number: 44

Principal Investigator: Ruth L. Kirschstein, M.D.

Other Investigators: Alan Rabson, M.D., NCI
Ronald Clark, Ph.D., LP-DBS
Amos E. Palmer, D.V.M., LP-DBS
Elizabeth Peters, LP-DBS
Kendall Smith, Ph.D., LBB-DBS
K. K. Takemoto, Ph.D., NIAID
Harvey Ozer, M.D., NIAID
Harold Baer, Ph.D., LBP-DBS
Sotiros Chaparas, Ph.D., LBP-DBS
Lewellys F. Barker, M.D., LVR-DBS, LVI-DBS
Carolyn Hardegree, M.D., LBP-DBS

Cooperating Units: Pathologic Anatomy Branch, NCI
Laboratory of Biophysics & Biochemistry, DBS
Laboratory of Biology of Viruses, NIAID
Laboratory of Bacterial Products, DBS
Laboratory of Virology & Rickettsiology, DBS

Man Years:
Total: 7.0
Professional: 1.8
Other: 5.2

Project Description:

Objectives: Experimental infectious diseases are studied as to pathogenesis in the hope that this may further elucidate the pathogenesis of infectious diseases in man and lead to their prevention.

Methods Employed: Infectious agents and related substances or substances causing an immunologic response are injected into a variety of animals when newborn or adult, and gross autopsies and histologic examinations are performed at timed intervals so that the development of the disease process and the pathologic changes may be studied.

Major Findings: (1) (With Dr. Rabson and Mrs. Peters) Studies on the comparative oncogenic effects of polyoma virus strains, simian virus 40, and adenoviruses are continuing. The oncogenic potential of these viruses can be enhanced by decreasing the immunologic capability of these animals. The effect of various drugs and chemicals on the oncogenic potential and on the lethality of these viruses for various species of animals is under investigation.

(2) (With Dr. Rabson and Mrs. Peters) Studies of cultured cells from lymphomas of the "Burkitt" type and of viruses found by others to be associated with them are in progress. These cells have a limited ability to grow in heterologous hosts.

The pathogenesis of Herpes simplex virus infection in newborn hamsters and mice after various routes of inoculation has been studied. Despite the association of "herpes-like particles" with the "Burkitt" type lymphoma, Herpes simplex had no effect on the lympho-reticular system. Attempts to alter its infectivity and decrease the immediate mortality are underway, using a variety of chemical and physical means.

(3) (With Dr. Rabson) Studies of the ability of the oncogenic viruses to "transform" hamster cells and monkey cells in cell cultures are continuing. The cellular transformations have characteristics of malignancies and have varying growth potential when inoculated into animals, depending on the species. Attempts to alter this growth potential by changing immune response and using other target organs are continuing.

(4) (With Drs. Takemoto and Ozer) Studies on the immunologic, oncogenic, and cytopathic effects of various SV40 plaque strains are underway. Such strains have different oncogenic potentials both in vitro and in vivo.

(5) (With Dr. Hardegree) Studies of the pathologic reaction in muscles and ears of rabbits to mineral oil and Arlacel A (mannide monoleate) indicate that while each causes a granulomatous reaction, that caused by Arlacel A is much more severe. However, the free fatty acids (considered by some to be the toxic element in the Arlacel A) do not cause the same severe reaction that the Arlacel A does.

(6) (With Dr. Smith) The oncogenicity of adenovirus 12 for newborn hamsters is partially inhibited by the presence of adeno-associated virus (AAV). Thus, 60 per cent of newborn animals inoculated with adenovirus 12 alone developed tumors whereas only 19 per cent of those inoculated with adenovirus 12 and AAV had tumors. The incubation period was also prolonged in those hamsters inoculated with adenovirus 12 and AAV.

(7) (With Dr. Chaparas) Studies of the development of antibody-producing cells in the lymph nodes of guinea pigs required the use of specialized stains. These studies are complicated by the natural flora of the animals which alters the production of antibodies. Inoculation of B.C.G. in adjuvant produces different reactions, depending on the sensitization state of the guinea pigs.

(8) (With Drs. Palmer and Barker) Studies of the pathogenesis of epidemic typhus fever in monkeys have been completed. Monkeys are susceptible to the disease and the lesions produced are similar to but milder than those in man when the Breinl strain is used. Previous inoculation of the "E" strain is protective against challenge with the Breinl strain.

(9) (Dr. Palmer and Dr. Tauraso) Studies to detect and characterize the agent responsible for an epidemic of a febrile hemorrhagic disease among monkeys in the quarantine unit in 1965 are completed. To date, no other host has been found susceptible to the agent.

Significance to Bio-Medical Research and the Program of the Division: These studies are designed to elucidate aspects of the pathogenesis of infectious diseases in animals so that this model may serve to help provide the means of prevention of such diseases in man.

Proposed Course of Project: It is expected that this program will expand in 1968-69.

Honors and Awards: None

Publications:

Barker, L. F., Palmer, A. E., Kirschstein, R. L., Hopps, H. E., and Patt, J. K., Experimental Infection with Rickettsia Prowazeki in Rhesus and Vervet Monkeys. J. of Infectious Diseases, 117:285-291, 1967.

Kirschstein, R. L., Smith, K. O., and Peters, E. A., Inhibition of Adenovirus 12 Oncogenicity by Adeno-associated Virus, Proc. Soc. Exp. Biol. and Med. (in press).

Hardegree, M. C. and Kirschstein, R. L., The Toxicity of Free Fatty Acids and Arlacel A. Ann. of Allergy (submitted for publication).

Palmer, A. E., Allen, A. M., Tauraso, N. M., and Shelokov, A., Simian Hemorrhagic Fever. I. Clinical and Epizootiological Aspects of an Outbreak among Quarantined Monkeys. Am. J. Trop. Med. & Hyg., May, 1968.

Allen, A. M., Palmer, A. E., Tauraso, N. M., and Shelokov, A., Simian Hemorrhagic Fever. II. Pathology. Am. J. Trop. Med. & Hyg., May, 1968.

Tauraso, N. M., Shelokov, A., Palmer, A. E., and Allen, A. M., Simian Hemorrhagic Fever. III. Isolation and Characterization of a Viral Agent. Am. J. Trop. Med. & Hyg., May, 1968.

Serial No. DBS 45
1. Pathology
2. Histopathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Histologic and Ultrastructural Development of the Central Nervous System and its Relationship to the Immunological Response

Previous Serial Number: 45

Principal Investigator: Ronald G. Clark, Ph.D.

Other Investigators: Philip McGrath, LP-DBS
Joyce Johnson, LP-DBS
Charles Manclark, Ph.D., LBP-DBS
Walter Stanley, Ph.D., LP-NIMH
Edward Bacon, Ph.D., LP-NIMH

Cooperating Units: Laboratory of Bacterial Products, DBS
Laboratory of Psychology, NIMH

Man Years:
Total: 2.0
Professional: 1.0
Other: 1.0

Project Description:

Objectives: To ascertain the normal developmental processes in the nervous system of laboratory animals and to relate ultrastructural alterations of these developmental processes in experimental animals in response to immunologic stimulation.

Methods Employed: In utero and postnatal animals are sacrificed at timed intervals, and gross evaluation and histologic examinations are performed to determine the normal development of the central nervous system. The histologic evaluation involves both light and electron microscopy. Experimental animals are studied similarly, and microscopic as well as ultrastructural deviations from the normal are noted and evaluated.

Major Findings: Light microscopy studies of the neuroanatomical development of hamsters, rabbits, and guinea pigs have been completed, and extensive studies of other animal species, including the Rhesus monkey and beagle dog, are in progress. Electron microscopy studies of myelinization

in the central nervous system of normal rabbits are complete. Studies concerning the possible adjuvant effect of pertussis vaccine (when combined with complete Freund's adjuvant and CNS tissue) in inducing experimental allergic encephalomyelitis have recently been initiated with LBP-DBS.

Significance to Bio-Medical Research and the program of the Division: These studies are closely related to the problem of post-vaccinal encephalitis and the production of vaccines in tissue from the central nervous system of animals.

Proposed Course of Project: The endeavor will continue with emphasis being placed on the study of ultrastructural changes in relation to immunological response.

Honors and Awards: None

Publications: None

Serial No. DBS 95
1. Pathology
2. Animal Testing and Histopathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Development of Toxic States

Previous Serial Number: 95

Principal Investigators: Amos E. Palmer, D.V.M.
Ronald G. Clark, Ph.D.

Other Investigators: Carolyn Hardegree, M.D., LBP-DBS
Pinya Cohen, Ph.D., LCA-DBS
Roberta Gardner, Ph.D., LBP-DBS
Howard Bohner, LAB-DRS

Cooperating Units: Laboratory of Bacterial Products, DBS
Laboratory of Control Activities, DBS
Laboratory Aids Branch, DRS

Man Years:
Total: 0.3
Professional: 0.3
Other: 0.3

Project Description:

Objectives: To determine the toxicity of various biological products.

Methods Employed: The materials are inoculated into a variety of animals. Gross autopsies and histologic examinations are performed at timed intervals so that the development of the toxic process and the related pathologic changes can be evaluated. In addition, physiologic, hematologic, chemical, and clinical observations are studied.

Major Findings: (1) (With Dr. Hardegree) In vivo hemolysis is produced in rhesus monkeys when the hemolytic fraction of tetanus toxin is injected. Electrocardiographic studies performed on these animals are erratic and not reproducible. Gross and histologic necropsy examinations have been unrevealing.

(2) (With Dr. Cohen) Studies of the pathologic changes induced by the supposed neurotoxic component in coral snake venom have been halted.

(3) (With Dr. Gardner and Mr. Bohner) Studies comparing the toxicity of various pertussis vaccines in germ-free and conventional mice have been halted.

Significance to Bio-Medical Research and the Program of the Division: The results of this study may be significant in making it possible to reduce the toxicity of biological products used in man.

Proposed Course of Project: This project was terminated when one of the principal investigators resigned in October, 1967 and two of the other investigators changed their assignments.

Honors and Awards: None

Publications: None

Serial No. DBS 96
1. Pathology
2. Animal Testing
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Breeding of Simian Primates

Previous Serial Number: 96

Principal Investigators: Amos E. Palmer, D.V.M., LP-DBS
William Tullner, Ph.D., LB-NICHD

Other Investigators: Charles Turner, LB-NICHD
Donald Barber, LB-NICHD
Ben Kittridge, LB-NICHD

Cooperating Units: Laboratory of Biology, NICHD

Man Years:
Total: 0.6
Professional: 0.1
Other: 0.5

Project Description:

Objectives: To develop techniques to detect the stage of the estrus cycle, period of gestation, and for semen collection, dilution, storage and artificial insemination of monkeys in an effort to increase the efficiency of reproduction.

Methods Employed: Semen is collected by electroejaculation. Liquid portions of ejaculates are evaluated for sperm concentration, motility, and total content. Dilution is performed using one of several extenders and the samples are frozen in liquid nitrogen or used to inseminate females. Cytologic and chemical methods have been used to detect estrus and early pregnancy.

Major Findings: Electroejaculation has regularly produced semen samples of relatively consistent quality. Dilution of the liquified semen permits storage at refrigerator temperature for reasonable periods of time. Freezing in liquid nitrogen permits storage of viable spermatozoa for prolonged periods of time. Losses vary depending on diluent used prior to freezing. Several females have been inseminated without conception to date. As a result of normal breeding procedures, 9 infant monkeys have been born

in the DBS colony during the past year. A technique of electroejaculation by phallus stimulation has just begun, which yields samples of high quality with regularity.

Significance to Bio-Medical Research and the Program of the Division: This project is an effort to increase the supply of gravid females and/or newborn infant monkeys which are acutely needed for research projects, but are unavailable from commercial sources.

Proposed Course of Project: This project was terminated when the principal investigator resigned in October, 1967.

Honors and Awards: None

Publications: None

Serial No. DBS 112
1. Pathology
2. Animal Testing and Histopathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Developmental Studies for Control Testing of Attenuated Live Virus Vaccines

Previous Serial Number: 112

Principal Investigator: Ruth L. Kirschstein, M.D.

Other Investigators: Ronald G. Clark, Ph.D., LP-DBS
Amos E. Palmer, D.V.M., LP-DBS
Harry Meyer, M.D., LVI-DBS
Paul Parkman, M.D., LVI-DBS
Hope E. Hopps, LVI-DBS
Alexis Shelokov, M.D., LVR-DBS
Nicola Tauraso, M.D., LVR-DBS
Edward B. Seligmann, Ph.D., LCA-DBS
Kendall Smith, Ph.D., LBB-DBS

Cooperating Units: Laboratory of Viral Immunology
Laboratory of Virology and Rickettsiology
Laboratory of Biophysics and Biochemistry
Laboratory of Control Activities

Man Years:
Total: 3.0
Professional: 0.4
Other: 2.6

Project Description:

Objectives: To gain information concerning the pathogenesis of rubella virus, mumps virus, and adenovirus infections in monkeys so that safety tests for attenuated live virus vaccines can be developed.

Methods Employed: Rhesus monkeys are inoculated with both wild and attenuated virus preparations, using various routes of inoculation depending on the mode of action of the particular virus. Animals are observed for evidence of disease and specimens of blood, and various secretions are taken for evidence of virus multiplication. Uninoculated animals may be placed in cages with inoculated animals or in the same room in order to study transmissibility of the wild or attenuated infection. At the end of the observation

period which is 17-30 days, depending on the virus and the route of inoculation, all animals are bled to determine antibody levels. The monkeys are then sacrificed, and various organs are assayed for presence of virus. All organs from each animal are examined histologically for evidence of abnormality.

Major Findings: (1) (With LVI) Studies of the pathogenesis of rubella virus infection in monkeys are continuing. To date, the virus has been found to multiply in the monkey but not to produce any pathologic alterations. Investigation of communicability of various strains of rubella virus from monkey to monkey is underway, using various routes of inoculation.

(2) (With LVI) Studies of the pathogenesis of mumps virus infection in monkeys indicate that these animals do develop neutralizing antibody after inoculation, but that no virus can be recovered from any tissues. No differences between a wild and an attenuated strain could be noted. Despite the lack of evidence of viral replication in the central nervous system, certain pathologic changes appear to occur consistently when the virus is inoculated intrathalamically. These lesions appear to be unrelated to neurovirulence of this virus for man.

(3) (With LVR) Attempts to infect monkeys with human adenoviruses are underway, but the final results are not yet available.

(4) (With LBB) It has been known for some time that human adenoviruses will cause a choroiditis and ventriculitis in brains of monkeys inoculated intracerebrally with human adenoviruses. Attempts are being made to elucidate this phenomenon by use of certain specific fractions of the crude virus preparations prepared by cesium chloride density gradient centrifugation. It appears that purified virus particles will cause the pathologic changes. Further investigation is in progress.

(5) (With LVR and LCA) Studies of Yellow Fever Vaccine (17D strain) after removal of the contaminating avian leucosis virus indicate that its neurovirulence has not been altered.

Significance to Bio-Medical Research and the Program of the Division: These studies are designed to develop information which will allow control testing of new viral vaccines to be meaningful and assure safety of the product. The Additional Standards for Mumps Virus Vaccine have been based on these studies.

Proposed Course of Project: It is expected that this program will expand during the next years as more new vaccines are developed.

Honors and Awards: None

Publications:

Parkman, P. D., Meyer, H. M., Jr., Hopps, H. E., and Kirschstein, R. L., Laboratory Studies with an Attenuated Rubella Virus, Presented at the First International Conference on Vaccines against Viral and Rickettsial Diseases of Man, Pan American Health Organization, Washington, D. C., Scientific Publication No. 147, pp. 381-389, 1967.

Serial No. DBS 113
1. Pathology
2. Histopathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: In vitro Study of Cells from the Central Nervous System

Previous Serial Number: 113

Principal Investigator: Ronald G. Clark, Ph.D.

Other Investigators: Joyce Johnson, LP-DBS

Cooperating Units: None

Man Years:
Total: 0.7
Professional: 0.2
Other: 0.5

Project Description:

Objectives: (1) To perfect methods of identification of cell types (astrocytes, oligodendrocytes and neurons) in tissue culture from the central nervous system. (2) To grow pure cultures of individual cell types from the central nervous system.

Methods Employed: Normal animals are sacrificed and specific areas of the brain aseptically removed for tissue culture. After removal, the tissue is treated in one of three ways: (1) untreated explants are grown in tissue culture, (2) lightly trypsinized tissue is grown in tissue culture, and (3) untreated tissue is ultrasonically vibrated and then grown in tissue culture.

After growth in tissue culture, the cell sheets (on coverslips) are removed and treated with special stains (gold and silver techniques). These stains, normally used on frozen sections of whole brain, will have to be modified in order to adequately characterize the cells.

Major Findings: Project is in its initial stage and has been slow to start while techniques are being developed.

Significance to Bio-Medical Research and the Program of the Division: The results of this study may enable the use of CNS tissue culture in studying the pathogenesis of neurotropic viruses.

Proposed Course of Project: This project will continue and expand in the following year.

Honors and Awards: None

Publications: None

Serial No. DBS 114
1. Pathology
2. Animal Testing
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of Tissue Transplants in Various Animals

Previous Serial Number: 114

Principal Investigator: Amos E. Palmer, D.V.M., LP-DBS

Other Investigators: Robert Davis, M.D., Surgery Branch, NCI
John Lewis, M.D., Surgery Branch, NCI
Kehl Markley, M.D., LBP-NIAMD

Cooperating Units: Surgery Branch, NCI
Laboratory of Biochemical Pharmacology, NIAMD

Man Years:
Total: 1.0
Professional: 0.5
Other: 0.5

Project Description:

Objectives: To study the effect of thymectomy and/or treatment with antilymphocyte serum, antithymocyte serum or other therapeutic agents upon host response to homografts in rabbits and monkeys.

Methods Employed: Thymectomized and intact animals are treated with the various antisera or chemotherapeutic agents utilizing various routes and schedules. These animals are then given tissue transplants and the host response to these transplants evaluated visually by radiographs and biopsies for histologic evaluation.

Major Findings: (1) (With Drs. Davis and Lewis) Skin homografts in rhesus monkeys are retained approximately three times as long when the animals are treated with antilymphocyte serum as when no treatment is given. Thymectomy appears to have no effect.

(2) (With Dr. Markley) Studies of the effect of chemotherapeutic agents on skin homografts in rabbits are currently being evaluated.

Significance to Bio-Medical Research and the Program of the Division: Results from these studies may reveal means of extending the life of heterografts without completely eliminating recipient resistance to infection and yield information pertinent to the rejection of graft-versus-host phenomenon.

Proposed Course of Project: This project was terminated when the principal investigator resigned in October, 1967.

Honors and Awards: None

Publications: None

Serial No. DBS 133
1. Pathology
2. Animal Testing
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of Marmosets (Saguinus mystax) and their Suitability for Biologics Testing

Previous Serial Number: None

Principal Investigator: Douglas Lorenz, Ph.D., LP-DBS

Other Investigators: Ruth L. Kirschstein, M.D., LP-DBS
Marshall Ford, LP-DBS
George Rusten, LP-DBS

Cooperating Units: None

Man Years:
Total: 3.5
Professional: 1.0
Other: 2.5

Project Description:

Objectives: The goals of this program are: (1) to maintain a colony of marmosets under laboratory conditions and (2) to describe the microbiology of that colony. From such work, a corps of personnel will become familiar with maintenance methods unique to these animals; also, means of recognizing and controlling marmoset diseases and microflora will become available. Only after this baseline study has been completed can marmosets be considered as subjects for biologics testing programs.

Methods Employed: Eighty-five marmosets have been maintained in a 12 x 17 foot room where the mean temperature is 80°F. and the mean relative humidity is 50 per cent. Colony maintenance is performed by a restricted number of people to reduce contamination of the marmosets with agents from other primates. NIH monkey chow is the basic food. Each pair of animals is housed, at present, in a standard rabbit cage. The cages are steam-cleaned every three weeks.

Classical methods of diagnostic microbiology have been employed to isolate and identify ectoparasites, helminths, protozoa, fungi, bacteria, Mycoplasma, and viruses. Serum samples from each animal have been collected and stored at -20°C. Weights, rectal temperatures, and differential white blood cell counts were also obtained by classical methods.

Major Findings: The original colony consisted of 86 Saguinus mystax which were obtained from a vendor in Florida after a guaranteed 30-day conditioning period. The animals arrived September 27, 1967. To date, 79 per cent have survived and the rate of loss has decreased considerably from that of the beginning phases of the colony. Most deaths are attributed to heavy parasite infections.

Maintenance is proceeding smoothly. One major problem has been the incidence of injury to handlers during transfer or examination of the marmosets. The problem seems to have been solved by the use of heavier gloves. Another problem is the net loss of weight of animals in the colony. It has not been ascertained yet whether this loss is due to parasitism or nutrition.

All animals have been examined for blood parasites. Microfilaria were found in 83 per cent of the marmosets. Trypanosomes were found in 42 per cent of the animals.

All animals from which suitable specimens have been obtained demonstrate helminth ova in stool specimens. No effort has been made to speciate these, but specimens much like Trichuris sp. and Trichostrongylus sp. predominate. At autopsy, large masses of adult worms have been found in all animals.

In the intestine, bacteria of the Enterobacteriaceae were sought exclusively. Bacteria demonstrating biochemical reactions similar to those of Shigella sp. were found in 16 per cent of the animals. Two marmosets carry bacteria biochemically resembling Salmonella sp. Fifty-three per cent of the marmosets carry Proteus sp.

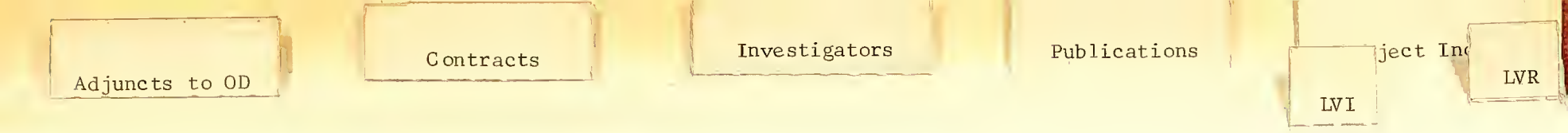
No enteric viruses have been recognized thus far by their cytopathogenic effects in primary rabbit kidney, primary Rhesus monkey kidney, or HEP-II cells.

Significance to Bio-Medical Research and the Program of the Division: This work will contribute to any evaluation of the marmoset as a subject for biologics testing. The small size of this primate would allow much more economical biologics testing.

Proposed Course of Project: In the coming year, efforts shall be directed toward improving the methods of handling the animals and increasing their weight. The surest way of decreasing the parasite burden of a colony of marmosets would be to initiate that colony in the laboratory. Methods of enhancing the breeding rate will be investigated. Meanwhile, experiments to evaluate various drugs to reduce or control parasites or pathogenic bacteria will be completed. Within the year, the microbiological profile of the wild marmosets will be completed. It is planned that recognition and control of common diseases in the marmosets will be achieved and that a nucleus of laboratory-bred marmosets will be available for further studies.

Honors and Awards: None

Publications: None


Adjuncts to OD
Contracts
Investigators
Publications
LVI
LVR

Annual Report of
Laboratory of Viral Immunology
Division of Biologics Standards
July 1, 1967 through June 30, 1968

Laboratory Chief's Summary

The Laboratory of Viral Immunology maintains a program oriented toward current or anticipated areas of need in the biologics field. Approximately two-thirds of the Laboratory's effort is devoted to research-associated activities. Other commitments include (1) control testing of products and (2) support of the Office of the Director in an advisory capacity on such matters as licensing, investigation of new drug applications, contract operations, reports of adverse effects related to the use of biologics, etc.

Since biologic products are used for the control of human illness the efforts of the Laboratory are most easily summarized in terms of the specific diseases to which they apply: Rubella. The Laboratory has maintained its position at the center of the international program to develop a vaccine to control and perhaps eradicate this important cause of crippling birth defects. The HPV-77 strain of rubella virus attenuated by our group and made available in May 1966 has been used by pharmaceutical companies in preparing approximately two hundred thousand doses of experimental vaccine. At present these preparations have been field tested in several thousand individuals and by the end of the current fiscal year the number inoculated should reach 20,000. Rubella vaccine will likely become commercially available during calendar year 1969.

Use of the rapid blood test (HI procedure) developed by the Laboratory in 1966 to assay vaccine-induced immunity has played a key role in advancing the date of expected completion of vaccine development from the early 1970's to 1969. This acceleration of the program should reduce vaccine development costs to the government alone by well over a million dollars. Because of their obvious commercial value, patent applications covering the vaccine and the HI test were filed by our Laboratory in 1966.

In recent months the Laboratory resolved several fundamental questions by developing highly modified strains of rubella virus and by producing and clinically testing experimental vaccines prepared from these viruses. It was shown that "overattenuated" virus was incapable of inducing a satisfactory immune response in man. Moreover, replication of the vaccine strain of virus in the respiratory tracts of recipients was essential to the production of protective antibodies. These data permitted determination of the exact degree of virus modification ideal for vaccine production.

Viruses replicate only in living cells and several options exist in the selection of animal species as donors of tissues for cell cultures used in rubella vaccine preparation. For evaluation of the advantages and disadvantages of these systems, experimental live rubella virus vaccines were produced by our Laboratory in primary monkey kidney and chick embryo fibroblast cell cultures and in primary dog kidney and duck embryo fibroblast

cell cultures by two pharmaceutical research teams. These experimental preparations, eight in all, were subjected to comparative clinical trials by our group. The results indicated that all of the vaccines were free of harmful effects. The attenuated viruses grown in monkey, dog and duck cells were fully immunogenic whereas chick embryo adaptation of the virus was shown to have resulted in a significant reduction in vaccine immunogenicity. From such studies it becomes possible to identify the optimum tissues for use in final vaccine development.

This past fall our Laboratory led in another critical step: initiation of the first experimental live rubella vaccine study in the open population. All earlier trials had been performed in institutions where rigid quarantine measures could be enforced. Our protocol for vaccine evaluation in family groups has since been used by others for extensive open population field testing.

Important preparations have been made for assuming biologics control responsibilities with rubella vaccines by: standardization and distribution of a reference serum, development of a sensitive immunofluorescent plaque technique for rapid assay of vaccine potency and determination of the value of new types of cell cultures for rubella virus isolation.

Finally basic investigations of the relationship between hemagglutinating particles and infectious virus were completed.

It has been a year of unsurpassed progress in the rubella field.

Mumps. A live virus vaccine for mumps became commercially available in January 1968. Anticipating this event, the Laboratory has conducted a mumps research program for more than three years. Our clinical trials with the Jeryl Lynn strain established that the attenuated virus cannot be detected in the saliva, blood or urine of vaccine recipients. Also these studies confirmed that use of the vaccine was not associated with adverse reactions in either adults or children. An ongoing investigation designed to determine the duration of vaccine-induced immunity has shown that one year after immunization the level of protective antibodies is unchanged. These data were made particularly meaningful by the inclusion of a control group that experienced natural mumps at approximately the same time that the test group was vaccinated.

The development of a laboratory "marker" system capable of distinguishing attenuated from virulent mumps virus and of a vaccine potency assay test further insured the smooth transition without delay from the phase of developmental research to commercial use of the new product.

More basic laboratory investigations have been concerned with central nervous system infections caused by mumps virus. In this work hydrocephalus could be produced regularly in newborn hamsters by the direct intracerebral inoculation of either virulent or attenuated virus. This experimental system is of particular note because of the growing scientific interest in the role of viruses in chronic disease states.

Smallpox. Although a discussion of smallpox immunization might seem inappropriate in describing modern vaccine research, such is not the case. Having retreated to endemic areas in Asia, Africa and South America, smallpox no longer exists in North America. Nevertheless, the continued need to immunize all American children results in 12 vaccine-associated deaths per year and many thousands of cases of lesser illness.

Viewing these problems our Laboratory has been interested in: (1) finding more efficient methods to immunize the citizens of the developing nations at direct risk to smallpox and (2) searching for safer ways to perform primary vaccination of populations in countries not exposed to smallpox.

As an outgrowth of research by the Laboratory on item number one, the first combined live measles-smallpox was licensed this year. This product which is given by jet injection should be particularly useful in overseas technical assistance programs.

The logical approach to item number two appears to be in developing a more attenuated vaccinia virus or a method of immunization less prone to induce undesirable reactions. Perhaps primary vaccination of the child could have sensitization rather than protective immunization as its goal. Infants sensitized to the virus might then be revaccinated with more immunogenic material but at considerably less risk.

Studies of the pathogenesis of vaccinia infections in monkeys provided data that guided the direction of a clinical trial using small doses of virus inoculated subcutaneously. The results were promising and are described in detail in the individual project reports.

Infectious Mononucleosis and Herpes-like Virus Infections.

Many groups have worked with a herpes-like virus present in certain strains of cells cultured from cases of Burkitt's lymphoma. The Laboratory's Section on Viral Genetics found that cultured leucocytes from several healthy donors in this country contained similar herpes-like virus particles. Using extremely sensitive serologic tests it was possible to show that antibodies to this agent are widespread in the population and bear no apparent relationship to the presence or absence of antibodies for herpes virus of man, i.e., herpes virus hominis (simplex). Related antibodies were very commonly found in sera of subhuman primates (except baboons) but not in specimens from common species of lower animals.

Recently, reports appeared giving evidence that herpes-like viruses of man caused infectious mononucleosis. Using sera provided by the group at Yale University our investigators were able to confirm the serological association of herpes-like virus infections and infectious mononucleosis.

Because of the theoretical possibility that infectious mononucleosis could be a sub-leukemic manifestation of infection caused by a human leukemia virus, this area of investigation is especially exciting.

Viral Hepatitis. A continuing effort is being directed toward the elucidation of the etiology of viral hepatitis. Investigators in the Section on Immunology identified two agents recovered by workers at the Johns Hopkins University as reoviruses. Studies in the Laboratory also provided results indicating that these reoviruses were not related to human hepatitis.

New approaches such as fluorescent techniques and cell fusion methods are being applied to specimens from hepatitis cases. A prospective study is being conducted in cooperation with another institute. Here, specimens are collected periodically from surgical patients who have received blood transfusions.

If and when the etiologic agents of viral hepatitis are cultured in the laboratory one can expect the beginning of a major effort to develop vaccines.

Diseases Caused by Genetic Defects. The Laboratory has maintained a small program concerned with cell culture studies of diseases caused by genetic defects. Diploid cells derived from selected tissues and maintained by serial propagation in culture retain certain metabolic characteristics of the parent tissue. Consequently, appropriate studies of these cells can provide information identifying a specific heritable disease or genetically determined predisease state of the donor. A novel use of this technique now being explored involves culture of cells obtained by amniocentesis to determine genetic flaws in the fetus during early gestation.

Vaccine-Related Cell Culture Studies. With the need to obtain detailed data regarding several types of animal cells potentially important in the study or production of vaccines, it was necessary to produce a variety of special cell cultures in the Laboratory. These figures are summarized elsewhere in the Laboratory's annual report.

For example, the Section on Immunology produced the equivalent of 30,000 tubes of avian leucosis-free primary chick embryo cell cultures in supporting the rubella research program. These cells were from eggs of several ages, from both whole embryo and selected embryo tissues and grown under varied environmental conditions. Such exacting requirements were necessary in determining the factors that would permit adaption of rubella virus to replication in chick cells and in the later production of our experimental chick embryo propagated rubella vaccines for clinical trial.

Each of the cell production efforts represents a special research project. Another example concerns the 7000 duck embryo cultures (tube equivalents) prepared by the Section. A pharmaceutical company proposes to manufacture a major live virus vaccine (rubella) in primary duck embryo cultures. Consequently, detailed information relating to chick cells was needed. Investigators in the Section produced and tested duck cultures on a weekly basis to obtain the necessary answers. Trial lots of experimental vaccine were prepared in duck embryo cells and virus growth characteristics determined. Primary cell cultures and duck fibroblasts maintained by serial transfer for up to 15 passages were studied for extraneous agents using cytopathic tests, the interference procedure and other appropriate techniques.

LABORATORY OF VIRAL IMMUNOLOGY

Control Program

July 1, 1967 through June 30, 1968

Antibody Assays: Using virus neutralization or hemagglutination-inhibition tests.

Immune Serum Globulin (Human)

Number of lots received	142
Poliovirus potency tests	96
Number of tissue cultures	20,160
Measles potency tests (HAI)	122
Number of titrations	244

Poliomyelitis Immune Globulin (Human)

Number of lots received	13
Potency tests	12
Number of tissue cultures	2,520

Measles Immune Globulin (Human)

Number of lots received	8
Potency tests (HAI)	8
Number of titrations	16

Testing of Reference Materials:

NIH Measles Reference Serum No. 1

Number of tests (HAI)	16
Number of titrations	32

NIH Reference Poliomyelitis Immune Globulin Lot 174

Number of tests for Polio antibodies	16
Number of tissue cultures	3,360

NIH Reference Poliomyelitis Immune Globulin Lot 175

Measles HAI tests	16
Number of titrations	32

Poliomyelitis Reference Serum IIA-4

Number of tests	19
Number of tissue cultures	4,750

Vaccinia Immune Globulin

Number of tests	6
Number of tissue cultures	180

Testing of Vaccines, Live, Attenuated:

Poliovirus, Oral

Monkey neurovirulence tests	28
Number of pre-inoculation sera	980
Number of tissue cultures	6,860

Testing of Vaccines, Inactivated:

Diphtheria, Tetanus, Pertussis and Poliomyelitis

Number of lots received	8

Poliomyelitis, Aluminum Phosphate adsorbed

Number of lots received	2

Poliomyelitis

Number of lots received	13
Number of monkey safety tests	3
Pre-inoculation sera	60
Post-inoculation sera	70
Number of tissue cultures	3,780
Monkey potency tests on polio component	5
Pre-inoculation sera	60
Post-inoculation sera	60
Number of tissue cultures	8,640

Potency and Marker Tests (Live, Attenuated Vaccines):

Poliovirus, Oral

Number of lots received	53
Monovalent lots tested for virus content and genetic markers	18
Trivalent lots test for virus content of each type	14
Number of tissue cultures	4,000

Measles

Vaccine lots	18
Reference (DBS)	30

Measles-smallpox

Vaccine lots	4

Mumps

Vaccine lots	45
Reference (DBS)	35

LABORATORY OF VIRAL IMMUNOLOGY

Production of Cell Cultures for Use in Control and Research Activities

July 1, 1967 through June 30, 1968

	Tubes	2 oz.	8 oz.	32 oz.
BS-C-1				
Measles potency	4500			
Mumps	2500			
Rubella vaccine safety tests	300			90
Research projects or outside distribution	2700	120	18	28
Vero				
Research	3300		80	15
RK-13				
Research	500			
BHK-21				
Research		100	50	34
Primary Chick				
Research	6240	1637	160	200
Primary Duck				
Research	3640	517	4	
Sirc				
Research		200	50	
Human Leukocytes				
Research			120	
TOTAL	23,680	2574	482	367

CONTRACT NARRATIVE
LABORATORY OF VIRAL IMMUNOLOGY
Fiscal Year 1968

Section on Viral Immunology

BALTIMORE BIOLOGICAL LABORATORY (PH-43-66-1163)

Title: Preparation of Mycoplasma Antigens and Antisera

Contractor's Project Director: Theodore R. Carski, M.D.

Project Officers (DBS): Hope E. Hopps, LVI
Michael F. Barile, LBP

Objectives: (1) To prepare standardized Mycoplasma antigens and antisera which will be available for use by the Laboratory of Bacterial Products and other research groups within the DBS; (2) to provide a testing service for detection of Mycoplasma in biological materials being used at the DBS, e.g.; tissue cultures, virus and rickettsial seed pools, etc.; and (3) provides services for isolation and identification of Mycoplasma from specimens obtained from other DBS Contracts (see PH-43-67-1100 and PH-43-67-1106).

Since the Research Reference Reagents Branch (NIAID) has a similar contract with the BBL for production of Mycoplasma antigens and antisera for the major human-derived strains, a cooperative program was set up. The DBS contract is concerned with reagents for the animal strains -- dog, swine, bovine, etc. An exchange of reagents is being made between the 2 contractors.

Methods Employed: Each strain of Mycoplasma, animal and human, will be cloned and certified by serologic identification prior to the preparation of immunizing antigens. Burros and/or donkeys will then be immunized with each of the antigens and both pre- and post-immunization sera, lyophilized, will be available.

Major Findings: (1) The DBS has received serum specimens from animals immunized with M. laidawii A & B, M. arginini, M. granularum, M. gallisepticum, M. spumans, M. hyorhinis, M. neurolyticum, (approximately 70 specimens); (2) conjugated antisera for use in fluorescent antibody studies M. orale, type I, M. orale, type II, M. arthriditis, M. laidlawii, M. gallisepticum, M. granularum, M. hyorhinis, M. pulmonis, M. spumans and T strain, ET77. (3) 422 specimens were submitted for either isolation or identification.

Significance to DBS Program and Bio-Medical Research: The Mycoplasma constitue an important group of organisms, both with immediate regard to the regulatory control of vaccines and to their overall significance in human disease.

Proposed Course of Project: This project was amended in September 1967 and will be considered for renewal.

Date Contract Initiated: September 28, 1966
Amended September 28, 1967

Current Annual Level: $130,202.

Serial No. DBS 49
1. Laboratory of Viral Immunology
2. Section on General Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigations concerning rubella virus and vaccine.

Previous Serial Number: DBS 49

Principal Investigator: Paul D. Parkman, M.D.

Other Investigators: Hope E. Hopps, M.S.
Thomas E. Hobbins, M.D.
H. Elliott Larson, M.D.
Judith P. Hamilton, B.S.
Barbara C. Meyer, M.S.
Joan C. Enterline, A.B.
Robert D. Douglas
Eva R. Brown, B.S.
Harry M. Meyer, Jr., M.D.

Cooperating Units: Laboratory of Biophysics and Biochemistry, DBS
Laboratory of Pathology, DBS
Walter Reed Army Medical Center, Department of Pediatrics

Man Years:

Total:	13.2
Professional:	7.3
Other:	5.9

Project Description: Part I. Studies of the biological characteristics of rubella virus.

Adaptation of Rubella Virus to Avian Cells.

Extensive clinical experience has provided documentation of the safety of chick embryo cells as a substrate for vaccine production. This has stimulated interest in preparing rubella virus vaccines in avian cells and numerous attempts to adapt the virus to avian cell cultures. Hilleman first reported the preparation of experimental vaccines from rubella infected duck embryo cell cultures. Following this, in our laboratory, experiments were designed to define the variables influencing adaptation of rubella virus to both duck and chick embryo cell cultures. Since convincing evidence for adaptation can only be demonstrated by evidence that virus will multiply in serial passage, the effect of each variable was examined during a series of 4-15 subcultures in either duck or chick embryo cells. The most important variables were found to be concerned with the incubation conditions of the infected cells, the time interval at which viruses were subcultured and the passage level of the

infecting virus. Virus growth was readily demonstrable in cultures held at 29°C or 32°C; at 35°C, however, lesser amounts of multiplication were observed, and serial propagation was not achieved. Growth was also enhanced by incubation in a roller apparatus. Maximum infectivity titers in rolled cultures were from 10- to 100-fold higher than those of cultures held in stationary racks. Virus yields from infected cultures were consistently higher at 14 and 21 days after inoculation than yields obtained at 7 days. In fact, in early studies, serial propagation of virus beyond the 5th passage was not obtained in cultures subpassaged at 7 day intervals. The passage level of the infecting virus in primary African green monkey kidney cells had a distinct effect on adaptation to avian cell cultures. While both low passage virulent and high passage attenuated rubella viruses propagated well in duck embryo cells, low passage virus was distinctly slower in attaining maximal titers. In one experiment, a series of four duck embryo cell cultures were required before the yields of virulent virus equalled those of 120th passage virus. In adapting rubella virus to chick embryo cell cultures this relationship was even more striking. While inoculation of chick embryo cultures with 5th African green monkey kidney (GMK) passage level virus produced significant multiplication in the first chick embryo passage, the virus produced failed to grow in subsequent passage. Higher GMK passages of virus showed an increasing propensity for multiplication. Thus 120th passage virus persisted throughout the first four chick embryo cell passages, although titers steadily diminished with each subculture, and adaptation was not achieved in any practical sense. Successful propagation was attained, however, with very high GMK passage virus. Using the attenuated vaccine strain passaged 150 times in GMK a consistent pattern of growth was observed. After an initial period of diminishing yields during the 1st 3 consecutive passages, rather striking increases in titer were observed at 4th and 5th passage. From the fourth to the fifteenth chick embryo passage the virus has appeared to be completely adapted; cultures have consistently yielded maximum infectivity titers ranging from $10^{3.5}$ to $10^{4.8}$/ml. These levels of virus production, however, were lower than those of rubella virus preparations produced in duck embryo, GMK and canine kidney cell cultures. To determine the feasibility of large scale production, 5 lots of experimental vaccine in 8 to 12 liter volumes were prepared in chick embryo cells grown in round 1 liter glass bottles incubated at 32°C on a roller apparatus. Virus titers in the system were maximal by the 10th day post-inoculation and allowed the production of vaccines containing more than 1000 TCD_{50}/0.5 ml human dose. These chick embryo cell culture grown viruses exhibited the <u>in vivo</u> and <u>in vitro</u> marker characteristics of the parent attenuated rubella virus strain, including immunogenicity for the rhesus monkey. These data suggested that these preparations might be immunogenic for man.

Development of a Fluorescent Plaque Assay for Rubella Virus.

A rapid and precise quantitating technique for rubella virus infectivity was developed using a fluorescent plaque assay in continuous RK_{13} rabbit kidney cell cultures. Foci of infected cells exhibiting granular, cytoplasmic fluorescence were counted 72 hours after infection when no morphologic changes could be seen by bright field microscopy. This technique provided several advantages: (1) a high degree of precision, (2) rapidity, and (3) ease of performance. In contrast to other plaque assays for rubella virus infectivity which also depend on interference or cytopathic effect, the new technique is equally sensitive to virus strains regardless of this cell culture passage history.

Comparison of Primary and Continuous Simian Cell Cultures for Use in Rubella Virus Isolation.

The routine procedure employed in this laboratory for isolation of rubella virus from clinical specimens is the primary green monkey kidney (GMK) -- ECHO-11 interference system; inoculated GMK cultures are challenged with ECHO-11 virus after a first and second serial passage of the specimens. The principal disadvantage of this method is the presence of extraneous viruses, indigenous to the kidney cells. If such agents are present they may destroy the cultures or multiply to sufficient titer to interfere with ECHO-11 virus; in either case the entire test must be repeated.

The report of Liebhaber et al (PSEBM 125: 636-643, 1967) described the use of a continuous GMK cell line (Vero) from rubella virus isolation studies. Here, the first passage of the specimens is carried out in Vero cells and the second passage is made in GMK, thus decreasing the risk of contamination with other viruses. The data indicated that if the second passage was made after holding the Vero cells for 21 days rather than 10, the isolation rate was increased significantly. The authors found it necessary to use GMK for the second passage since rubella-infected Vero cells do not interfere with ECHO-11 virus nor do they ordinarily exhibit cytopathic change.

Recently, the 2 isolation systems were compared in this laboratory. In 4 experiments, 93 coded specimens were tested simultaneously in each cell system. A total of 47 isolations was made. However, only 31 of these 47 isolations were made in both systems; of the remaining 16 positive specimens 8 were detected in GMK/GMK and the remaining 8 were in Vero/GMK. Thus, the frequency of positive isolations was essentially the same in each system. In our hands, the number of positive isolations has not been increased by maintaining the first passage material for 21 days in Vero cells. These studies will be continued during the coming year.

Project Description: Part II. Studies of rubella virus infection in man.

Since our original clinical trials with the HPV-77 strain of attenuated rubella virus continued investigations in our laboratory and by other groups have resulted in a stepwise accumulation of data concerning the behavior of the attenuated infection in man. More than 500 persons have received these experimental vaccines in studies completed thus far. Clinical trials currently in progress have increased this number into the thousands. In completed trials, the HPV-77 experimental vaccine has been tested in 200 susceptible persons; the derivative passaged 5 times in duck embryo cell culture was used in 286 persons; HPV-77 plus 12 canine kidney cell passages in 40 and HPV-120, a vaccine prepared in GMK at the 120th passage level, in 27 volunteers. In addition, most recently a group at 12 children was given HPV-150 passed 10 times in chick embryo cell cultures. None of these vaccinees have experienced any symptoms attributable to the attenuated virus inoculation and of this group of more than 500 persons, 96% have developed protective antibodies. None of an approximately equal number of control children maintained in intimate contact with the vaccinees has developed rubella virus antibodies as a result of their exposure. Thus, all evidence to date has indicated that HPV-77 and its several derivatives are not communicable. Antibody studies on 50 HPV-77 vaccinees and 16 persons with

natural rubella have provided a basis for estimating the duration of immunity. Assay of HAI and neutralizing antibodies two years after infection indicated that while levels following the attenuated vaccines were lower, there was a parallel pattern of antibody persistence in both groups. Antibody titers at two years were unchanged from the values observed 4 months following infection or immunization.

All of these earlier studies were conducted in closed populations where strict quarantine of vaccinees and their contacts was feasible. Based on this uniformly favorable experience, i.e., no vaccine associated reactions and no communicability, rubella vaccine trials in open populations appeared to be a logical next step. A study was conducted in 11 families receiving their medical care through the Walter Reed Army Medical Center Out-patient Department. To be eligible a family had to contain two or more rubella-susceptible children as demonstrated by appropriate serologic procedures. One or more children in each family were vaccinated, and the uninoculated sibling(s) served as contact controls. Thus far in our studies, 16 children have been immunized, all without reactions, and none of their 16 susceptible contacts contracted the infection. Now that clinical studies have reached this second stage, larger studies with a more rapid accumulation of experience can be expected. Because cell culture passage led to development of attenuated rubella viruses, it seemed theoretically possible that additional passage might provide an attenuated strain that was not excreted from the nasopharyngeal tissues of vaccinees. Further modification of rubella virus was attempted by several groups. Comparative studies have been made of HPV-77 and Benoit strain derivatives experimental vaccines at several levels of attenuation. Vaccines were prepared in our laboratory in GMK and chick embryo cell cultures, at the Merck Institute for Therapeutic Research by Dr. Maurice Hilleman in duck embryo cell cultures, and by Mr. Samuel Musser, at Philips Roxane Laboratories in canine kidney cell cultures. Vaccine associated reactions and communicability to contacts were not observed in any of the studies. The HPV-77 and HPV-77 derivative vaccines prepared in the mammalian cell cultures were remarkably similar in immunogenicity, and pattern of virus shedding produced irrespective of the type of cell culture and virus passage level used for vaccine production. These materials evoked antibodies in 94-100% of recipients. The geometric mean titers of antibody two months after vaccination ranged from 1:46 to 1:68. Seventy-two to 92% of these vaccinees had virus-positive throat swabs at one time or another during the 28 day observation period. The duck embryo cell culture adapted rubella viruses included progressively higher passages of the Benoit virus strain (C, D and E level) and a derivative of HPV-77 which had received five passages in duck embryo cells. HPV-77 and Benoit C and D level materials exhibited properties similar to those of the HPV-77 viruses produced in mammalian cells; over 90% developed antibodies and 64 to 82% of persons developing antibody shed virus in the pharynx. Higher virus passage in duck embryo cells resulted in a continuing modification of the virus which resulted in a distinct loss in immunogenicity. The E level Benoit strain vaccine induced antibody formation in only 29% of vaccines, and the geometric mean antibody level (GMT) in those responding serologically was only 1:11.

The immunogenicity of vaccines prepared using the chick embryo cell culture adapted virus also was less than satisfactory. Only 9 of 12 vaccinees developed antibodies; the GMT of these subjects 2 months after inoculation was only 1:16. Even with the Benoit E level and HPV-150 chick embryo cell adapted virus strains,

however, pharyngeal virus shedding was observed in 50% and 67% of vaccinees, respectively. Thus, excessive modification or "over-attenuation" of rubella viruses can occur. For vaccine production it seems desirable to select viruses that represent an intermediate level of modification.

Project Description: Part III. Studies directed toward the standardization of serologic tests for rubella.

A rubella reference serum derived from pooled human serum was purchased on contract from Ortho Diagnostics. This material was filtered, dispensed in one ml amounts lyophilized and flame sealed in glass ampules by Wyeth Laboratories. A preliminary series of antibody titrations using both hemagglutination inhibition (25 tests) and neutralization (4 tests) have indicated geometric mean antibody levels of 1:78 and 1:44 respectively. This material is being made available as a reference for the standardization of neutralizing and HAI antibody test systems.

Studies concerning the rubella virus hemagglutination inhibition test have been continued. Since the removal of non-specific inhibitors of hemagglutination is a critical factor in obtaining reliable test results and since several laboratories had experienced difficulty in this regard with the kaolin treatment procedure the relative efficacy of two other treatment methods was evaluated. Known positive and negative sera were tested for HAI in parallel either untreated or after: (1) acetone extraction, (2) heparin-manganous chloride treatment, or (3) the standard kaolin treatment procedure. In parallel titrations of sera treated by the three methods, each proved satisfactory for removal of inhibitors. The acetone extraction procedure was technically more complex than the other procedures. The heparin-manganous chloride method was as simple as the kaolin procedure, and preparation of the reagents needed (1.0 M $MnCL_2$ and heparin, 5,000 units/ml) was less time consuming. This technique also simplified the treatment of undiluted sera. Failure of this method to remove all inhibitors from lipemic sera occasionally produced spurious false positive results, especially in older serum specimens which had been subjected to repeated cycles of freeze-thawing. Antibody levels in sera were not significantly different following any of the three methods; geometric mean antibody titers of the DBS rubella reference serum following treatment with kaolin or heparin-$MnCL_2$ or after extraction with acetone were 1:83, 1:112 and 1:91 respectively. Thus, while each procedure was effective in removing non-specific inhibitors for rubella virus hemagglutinin, none of these, however, obviated the necessity for carefully controlled conditions for detecting residual non-specific inhibitors of hemagglutination.

Project Description: Part IV. Studies of the antigenicity of inactivated preparations of rubella virus.

No work with inactivated rubella preparations was performed during this reporting peroid.

Project Description: Part V. Studies of possible rubella virus oncogenic effects.

Observations on hamsters inoculated as newborns with HPV-77 rubella virus

have been concluded. None of the hamsters developed symptoms or tumors. Thus, there was no evidence to date that rubella virus induces tumors in this animal species.

Project Description: Part VI. Studies of rubella virus infections in experimental animals.

Earlier observations indicated that differences existed in the antibody responses of experimental animals infected with 3rd to 5th passage virulent and attenuated high cell culture passage viruses. Essentially 100% of animals given either virulent or attenuated HPV-77 virus developed high titer of both neutralizing and hemagglutination-inhibiting (HAI) antibodies. Production of complement-fixing (CF) antibodies was produced in approximately 2/3 of animals infected with virulent virus, but was not seen following HPV-77 virus infection. Higher levels of antibody were produced in animals given the virulent virus; neutralizing antibodies were slightly more than 2-fold higher, and HAI antibodies nearly 10-fold higher. Since there were significant differences in the immunologic responses of animals given virulent and attenuated viruses, a detailed comparison of tissue culture passage level and HAI antibody titer response was made with higher passage derivatives of HPV-77 in GMK, canine kidney, duck embryo cell cultures and chick embryo cell cultures. Groups of animals were inoculated parenterally with virus preparations in from 1 to 7 individual experiments, the animals were tested serologically for the development of HAI antibody. All animals in each of the groups given HPV-77 and the HPV-77 derivatives in dog, duck or monkey cells (including the 120th and 151st GMK passage levels) seroconverted. Geometric mean antibody titers ranged from 1:60 to 1:50. There were no significant differences in the antibody levels of any of these groups. In contrast, only 93% of monkeys given 7th chick embryo passage of HPV-150 virus, and only 2 of 4 given the 10th chick embryo passage level experimental vaccine seroconverted. The antibody titers of these two groups of animals was only 1:84 and 1:16 respectively. In a more recent experiment a virus pool at the 10th chick embryo passage level containing 10,000 TCD_{50} of virus failed to immunize any of 5 susceptible animals. These date indicated that the level of modification of rubella virus appeared to be unaffected by either 43 or 74 additional GMK passages, or by 5 duck embryo or 12 canine kidney cell culture passages. In contrast, over-modification to the point at which antibodies were delayed and inconsistent in appearance was reached in as few as 10 chick embryo cell culture passages. This data suggested that this rapid over-modification of rubella virus for the rhesus monkeys might be reflected in over-attenuation for man. This correlation confirms the usefulness of the rhesus monkey system for studying the immunogenic potential of attenuated rubella viruses.

Significance to Biomedical Research and the Program of the Division:

All work pertains to problems concerned with the prevention of rubella and the control of rubella vaccine.

Honors and Awards:

Dr. Parkman and Dr. Meyer: E. Mead Johnson Award for Pediatric Research, 1967.

Dr. Parkman: United States Jaycees Ten Outstanding Young Men Award, 1967.

Dr. Parkman and Dr. Meyer: Invited to lecture at the Joseph P. Kennedy, Jr. Foundation Fourth International Scientific Symposium on Mental Retardation.

Dr. Parkman and Dr. Meyer: Invited to lecture at the American Association on Mental Deficiency Plenary Session.

Dr. Parkman: Invited to serve as chairman at the Special Section on Infectious Disease of the Society for Pediatric Research.

Publications:

Meyer, H.M., Jr., Parkman, P.D., Hobbins, T.E. and Ennis, F.A.: Clinical Studies with Experimental Live Rubella Virus Vaccines (Strain HPV-77) and an Evaluation of Vaccine Induced Immunity. Amer. J. Dis. Child. 127: 1968 (in press).

Amstey, M.S., Hobbins, T.E. and Parkman, P.D.: Density Gradient Ultracentrifugation of Rubella Virus. Proc. Soc. Exper. Biol. Med. 127: 1968 (in press).

Meyer, H.M., Jr.: Rubella Vaccine: A Review of Vaccine Development and Problems. Proceedings of the Fifth Immunization Conference, Immunization Program of the Communicable Disease Center, 1968 (in press).

Parkman, P.D. and Meyer, H.M., Jr.: Prospects for a Rubella Virus Vaccine. Perspectives in Medical Virology, vol. 12, 1968 (in press).

Parkman, P.D. and Meyer, H.M., Jr.: Development of Live Attenuated Rubella Virus Vaccines. Proce dings of the Joseph P. Kennedy, Jr. Foundation Fourth International Scientific Symposium on Mental Retardation. (in press).

Serial No. DBS 48

1. Laboratory of Viral Immunology
2. Section on Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigations Concerning Mumps Virus and Vaccine.

Previous Serial Number: DBS 48

Principal Investigator: Francis A. Ennis, M.D.

Other Investigators: Hope E. Hopps, M.S.
Robert D. Douglas
Thomas E. Hobbins, M.D.
Eva R. Brown, B.S.
Barbara C. Meyer, M.S.
Harry M. Meyer, Jr., M.D.

Cooperating Units: None

Man Years:

Total:	2.7
Professional:	1.9
Other:	0.8

Project Description: Part I. Studies directed toward the standardization of serologic tests for mumps.

In the 1966-67 Annual Report, information was given on the application of two mumps serologic procedures developed in this Laboratory. A large number of sera from patients with natural and attenuated mumps infections have been tested this year by these methods. Although Tween 80-ether treatment markedly enhances mumps hemagglutinin antigen titer, serum antibody results are similar to titers seen using a standard untreated mumps hemagglutinating antigen. The plaque neutralization test is the most sensitive indicator of low levels of specific antibody, and has been utilized effectively in screening for mumps susceptibility prior to vaccination, and determining the low antibody rises seen following vaccination with the live attenuated Jeryl Lynn strain. The hemagglutination-inhibition tests are less helpful when dealing with low levels of antibody because of widespread cross-reactions seen in this test with human sera at low dilutions.

Part II. Studies on virus excretion in natural mumps infection.

In last year's progress summary, a report was given on the lack of virus shedding in children vaccinated with the live attenuated Jeryl Lynn mumps strain. Reports in the literature state that in natural mumps virus could be isolated

from saliva up to one week following onset of parotid swelling, and from urine up to two weeks following onset of parotitis. No information was available as to the onset of virus excretion prior to clinical paratitis. This is of epidemiologic and biologic interest. Specimens of urine and saliva were obtained every other day from a group of children who were mumps susceptible and were exposed to a playmate with mumps. One child developed clinical mumps. There were no subclinical cases. Mumps virus was isolated from the saliva of this child one week prior to the onset of illness, and could be isolated from every saliva specimen up to 5 days after the onset of parotitis. Only one positive urine isolation was made on the eleventh day after onset of parotitis. The early appearance of virus in saliva supports early epidemiologic studies which revealed mumps to be contagious prior to the onset of clinical parotitis.

Part III. Clinical studies with natural and attenuated mumps infection.

In last year's Annual Report, there was described the initiation of a long term study at the Arkansas Children's Colony to evaluate protection against natural mumps and antibody persistence following vaccination with the live attenuated Jeryl Lynn strain. Of 41 vaccinees, 34 were found to be mumps susceptible on repeat testing. There was an epidemic of mumps occurring at the Children's Colony when the study was being done, and seven of the vaccinees developed unmodified natural mumps within one week after vaccination. A total of 36 children developed natural mumps, but these cases were isolated and had little contact with the vaccinee group.

The outbreak of natural infection afforded an opportunity to compare antibody formation following natural and attenuated infection. The two groups, 27 vaccinees and 36 naturally infected, have not been followed for one year. All the susceptible vaccinees developed mumps neutralizing antibody. The geometric mean titer is 10-20-fold less than that seen following natural infection, but is stable after one year. The same pattern of results are seen when the sera is tested by complement-fixation and hemagglutination-inhibition tests. None of the vaccinees, most of whom were post-pubertal, manifested any illness or complication after vaccination. Annual mumps antibody studies will be performed on these groups to determine the duration and level of mumps antibodies after natural and attenuated infections.

Part IV. Animal studies with natural and attenuated strains of mumps virus.

The first study compared infectivity of three mumps virus strains when given intranasally to guinea pigs. The strains employed were (1) Saunders strain passed 1 time in primary vervet kidney cells (VMK); (2) Saunders strain passed 25 times in VMK: (3) and the Jeryl Lynn strain. All three strains were immunogenic when given intranasally and none of the cage controls developed mumps antibodies.

The second animal study was done with hamsters. As Johnson reported, low passage mumps virus can induce hydrocephalus when injected intracerebrally into suckling hamsters.

Our studies with the Saunders 1 p VMK strain confirm their report. Hydrocephalus is also seen following intracerebral inoculation of suckling hamsters

with the Jeryl Lynn strain. Neutralized virus failed to induce hydrocephalus when inoculated intracerebrally. These studies are continuing using different animals and routes of inoculation.

Honors and Awards:

Dr. Ennis: Finalist in J. D. Lane Competition, given to outstanding young investigator in clinical research in U.S.P.H.S.

Dr. Meyer: Invited Chairman of session on Mumps and Mumps Vaccine 5th International Immunization Conference sponsored by NCDC. Held at Los Angeles, March 11-14, 1968.

Publications:

Ennis, F. A., and Jackson, D. Isolation of virus during the incubation period of mumps. J. Ped. 72:536, 1968.

Serial No. DBS 127
1. Laboratory of Viral Immunology
2. Section on Viral Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunological studies of viruses associated with human lymphoma and leukemia.

Previous Serial Number: DBS 127

Principal Investigator: Paul Gerber, Ph.D.

Other Investigators: Suzette M. Birch, B.S.
Edith N. Rosenblum, B.S.

Cooperating Units: John L. Smith Memorial Institute for Cancer Research, Maywood, New Jersey.
Immunology Branch, National Cancer Institute.

Man Years:

Total:	2.0
Professional:	1.0
Other:	1.0

Project Description:

Herpes-like virus (HLV) has been observed by several investigators not only in culture of Burkitt lymphomas and leukemic lymphocytes but also in leukocyte cultures derived from normal donors. The incidence and age distribution of antibodies to HLV was studied by means of a sensitive, quantitative CF test, and the relationship to known members of the herpes group of viruses was determined. In addition the incidence of antibodies among domestic and laboratory animals as well as subhuman primates was investigated.

Major Findings:

1. Antibodies to HLV are widely distributed among normal humans and subhuman primates, and are absent in lower animals.

2. Serological evidence indicates that primary infection with HLV may occur in young infants and possibly in some cases in utero.

3. CF antibodies to HLV persist for more than 10 years possibly for life, indicating persistent or recurrent infection.

4. About 50% of rhesus monkeys had CF antibodies to HLV at 1-4 days after their capture, suggesting enzactic maintanence of infection.

5. Recent findings indicate that CF antibodies to HLV develop during infectious mononucleosis, similar to the development of fluorescent antibodies observed by others.

Significance: This study is concerned with viral latency, and therefore of importance to the program of the DBS.

Course of Project: This project will be continued.

Publications:

Paul Gerber and Suzette Birch: Complement-fixing antibodies in Sera of Human and Non-human Primates to Viral Antigens Derived from Burkitt's Lymphoma Cells. Proc. Nat. Acad. Sc. (U.S.) 58: 478-484, 1967.

Paul Gerber and James H. Monroe: Studies on Leukocytes Growing in Continuous Culture Derived from Normal Human Donors. J. Nat. Can. Inst. 40: 855-866, 1968.

Paul Gerber, R.W. McCollum, D. Hamre, R.A. Moy and E.N. Rosenblum: Infectious Mononucleosis: Complement-fixing Antibodies to a Herpes-like Virus Associated with Burkitt Lymphoma. Science (in press).

Serial No. DBS 47
1. Laboratory of Viral Immunology
2. Section on Immumology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigations concerning the smallpox group of viruses and smallpox vaccines.

Previous Serial Number: DBS 47

Principal Investigator: Lewellys F. Barker, M.D.

Other Investigators: David P. Stevens, M.D.
Robert D. Douglas
Hope E. Hopps, M.S.
Barbara C. Meyer, M.S.
Harry M. Meyer, Jr., M.D.

Cooperating Units: Walter Reed Army Medical Center Pediatric Department

Man Years:

Total:	3.3
Professional:	1.6
Other:	1.7

Project Description: Part I. Study of immunity to vaccinia virus in experimental animals.

Studies to determine the effect of the route of inoculation and virus dose on antibody response and resistance to percutaneous challenge have been continued in rhesus monkeys. The infectious virus dose-response relationship has been investigated with the following findings. Percutaneous vaccination with material containing 10^7 to 10^9 $TCID_{50}$ per ml gives major reactions and resistance to challenge in the majority of vaccinated monkeys. A few animals vaccinated with material titering 10^7 had equivocal skin reactions followed by major reactions on revaccination. 10^3 and 10^5 $TCID_{50}$ per ml did not produce major reactions or resistance to challenge. No skin reactions followed subcutaneous injection of vaccine. Doses from 10^3 to 10^7 $TCID_{50}$ conferred partial immunity to percutaneous revaccination. Animals with antibodies did not always resist challenge.

Attempts to isolate virus frequently gave positive results in the draining nodes and spleens of animals vaccinated by the percutaneous route with 10^7 to 10^9 $TCID_{50}$ per ml. Almost no spread of virus from the inoculation site could be detected after subcutaneous injection of virus. Percutaneous vaccination with the further attenuated Rivers strain showed the same pattern of virus spread as seen with the DBS #2 reference lot of calf lymph vaccinia.

Project Description: Part II. Clinical studies.

A pilot study of the efficacy of primary subcutaneous injection of calf lymph vaccine was done at the Walter Reed Army Medical Center Pediatric Outpatient Department. There was no significant morbidity in 12 children inoculated with 10^3 to 10^7 $TCID_{50}$ of virus. Eight of the 12 developed antibodies. On percutaneous revaccination 2 months later, 4 of 11 children had major reactions, indicating partial efficacy of the subcutaneous injection. Further clinical studies to evaluate means of decreasing the morbidity and increasing the safety of routine vaccination are planned.

Significance to Bio-medical Research and the Program of the Division:

All of this work directly pertains to research problems in the prevention of smallpox and control of smallpox vaccine.

Honors and Awards: None

Publications: None

Serial No. DBS 53
1. Laboratory of Viral Immunology
2. Section on Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Use of tissue culture in the study and diagnosis of heritable metabolic disease.

Previous Serial Number: DBS 53

Principal Investigator: B. William Uhlendorf, Ph.D.

Other Investigators:

Cooperating Units: Laboratory of General and Comparative Biochemistry, NIMH
Laboratory of Molecular Diseases, NHI
Laboratory of Metabolism, NHI
Section on Human Biochemical Genetics, Arthritis and Rheumatism Branch, NIAMD
Laboratory of Neurochemistry, NIAMD
Laboratory of Biochemistry and Metabolism, NIAMD
Laboratory of Clinical Investigation, NIAID

Man Years:

Total:	2.0
Professional:	1.0
Other:	1.0

Project Description: The concept which is the basis of the subprojects listed below is that a disorder of an individual's metabolism may be reproduced in culture of his cells.

Methods which are standard in tissue culture, chemical analysis, enzyme assay, and radioactive tracer studies were used.

Objectives and Major Findings:

A. To develop procedures for culturing fetal cells from human amniotic fluid (AF). Twenty cell lines have been cultured from 26 samples of AF.

B. To determine the activity of several enzymes, known to be deficient in various heritable metabolic diseases, in AF cultures. AF cultures exhibit activity of sphingomyelinase, glucocerebrosidase, cystathionine synthase and cystathionase, suggesting that it may be possible to obtain the intrauterine diagnosis of Niemann-Pick disease (Types A and B), Gaucher's disease, homocystinuria and cystathioninuria.

C. To study the underlying defect in homocystinuria the enzyme affected

in this disease is demonstrable in cultured fibroblasts derived from normal human skin and is essentially lacking in cultures from skin of homocytinurics.

D. To delineate the biochemical defect in the sphingomyelinases (Niemann-Pick disease). Cell cultures from skin and bone marrow exhibit varying degrees of deficiency in sphingomyelinase activity, suggesting the use of tissue culture in the study of this disease complex.

E. To identify the enzyme defect in phytanic acid storage disease (Refsum's disease). By the use of cell cultures derived from skin biopsies of Refsum's patients the enzyme defect was shown to be in the α-hydroxylation of phytanic acid.

F. To study the defect in Chediak-Hagahi syndrome and the growth of several viruses in cells derived from patients with this abnormality. Cell lines have been obtained from four patients and six parents of patients. These cells are under study.

Significance to Bio-medical Research:

Human diploid cell culture is growing to be an important tool for the study of heritable metabolic diseases.

Proposed Course of Project: This project is continuing.

Honors and Awards:

Invited to lecture at the Joseph P. Kennedy, Jr. Foundation Fourth International Scientific Symposium on Mental Retardation.

Publications:

Steinberg, D., Herndon, J.H., Jr., Uhlendorf, B.W., Avigan, J., Mize, C.E. and Fales, H.M.: The enzymatic defect in Refsum's disease. J. Clin. Invest. 46: 1120, 1967.

Steinberg, D., Herndon, J.H., Jr., Uhlendorf, B.W., Mize, C.E., Avigan, J. and Milne, G.W.A.: Refsum's disease: Nature of the enzyme defect. Science. 156: 1740-1742, 1967.

Tsai, Su-Chen, Herndon, J.H., Jr., Uhlendorf, B.W., Fales, H.M. and Mize, C.E.: The formation of alpha-hydroxy phytanic acid from phytanic acid in mammalian tissues. Biochem. Biophys. Res. Commun. 28: 571-577, 1967.

Uhlendorf, B.W. and Mudd, S.H.: Cystathionine synthase in tissue culture derived from human skin: the enzyme defect in homocystinuria. Science (in press).

Serial No. DBS 137
1. Laboratory of Viral Immunology
2. Section on Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigations into the etiologic agent of viral hepatitis.

Previous Serial Number: None

Principal Investigator: David P. Stevens, M.D.

Other Investigators: Lewellys F. Barker, M.D.
Hope E. Hopps, M.S.
Eva R. Brown, B.S.

Cooperating Units: Surgery Branch, National Cancer Institute, NIH
Department of Pathobiology, Johns Hopkins University
School of Hygiene and Public Health

Man Years:

Total:	1.6
Professional:	1.4
Other:	.2

Project Description: Part I. Isolation attempts.

Tissue culture and fluorescent antibody techniques have been employed to evaluate clinical material obtained from patients with acute infectious and serum hepatitis in an effort to detect infectious agents.

Project Description: Part II. Identification of "candidate agents".

Several "candidate agents" -- uncharacterized infectious agents obtained by other investigators from patients with viral hepatitis -- have been studied in an effort to identify them.

Project Description: Part III. Prospective survey for serum hepatitis.

Surgical patients of the Surgery Branch, National Cancer Institute, who received blood transfusions are being followed prospectively to determine the incidence of anicteric and icteric serum hepatitis in this group. Clinical specimens obtained from these patients are being screened for the presence of infectious agents. Serial biweekly serum specimens are being obtained for viral serologic studies and for future use in evaluation of possible hepatitis isolates.

Objectives: The goal of these studies is the isolation and identification of infectious agents which are etiologic in viral hepatitis.

Methods Employed: Standard virologic and serologic techniques are used throughout the project.

Major Findings: Two candidate agents originally reported in 1961 by Hillis to produce cytopathic effect in chimpanzee kidney cultures both have been identified as reovirus Type 2. Studies have demonstrated no serologic association between this reovirus and viral hepatitis.

Significance to Bio-medical Research and the Program of the Division:

All of this work is pertinent to the goals of the DBS.

Honors and Awards: None

Publications: None

Serial No. DBS 46

1. Laboratory of Viral Immunology
2. Section on Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigations concerning measles virus and vaccine.

Previous Serial Number: DBS 46

Principal Investigator: Barbara C. Meyer, M.S.

Other Investigators: Hope E. Hopps, M.S.
Robert D. Douglas
Harry M. Meyer, Jr., M.D.

Cooperating Units: None

Man Years:

Total:	1.2
Professional:	0.8
Other:	0.4

Project Description:

The long term prospects for potency testing of live, attenuated measles vaccine led to a search for cell systems, in addition to BS-C-1, which might be used in assaying vaccines. The MA-134 line (embryonic Cercopitheus monkey kidney) appears to be a suitable substitute and has been tested extensively in the DBS and by 2 vaccine manufacturers. Recently, studies have been initiated with the Vero line of Cerocopitheus kidney. Attenuated measles virus produces a pronounced CPE in Vero cells which differs from that seen in measles-infected BS-C-1 and MA-134 cell cultures, where neither giant cell nor syncytia formation is a prominent feature. Instead, there is rounding of the cells with vacuole formation and eventual sloughing of the cell sheet. In comparative titrations of 26 samples, measles virus titered an average of 0.5 logs higher in Vero cells than in BS-C-1. Further quantitative comparisons of virus multiplication in Vero and BS-C-1 cell cultures are in progress.

Significance to Bio-medical Research and the Program of the Division:

This work relates directly to the measles vaccine control program.

Proposed Course of Project:

Studies of the characteristics of measles virus replication in mammalian cells will continue.

Honors and Awards: None

Publications: None

Serial No. DBS 52
1. Laboratory of Viral Immunology
2. Section on Viral Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Simian Virus$_{40}$.

Previous Serial Number: DBS 52

Principal Investigator: Paul Gerber, Ph.D.

Other Investigators: Edith N. Rosenblum, B.S.

Cooperating Units: None

Man Years:

Total:	2.0
Professional:	1.0
Other:	1.0

Project Description: Part I. Studies on virogenic SV$_{40}$-transformed cells.

Studies are in progress to determine the mechanism involved in the activation of the viral genome by fusion of tumor and indicator cells. Various aspects of this phenomenon are under investigation, e.g., fusion of several non-virogenic SV$_{40}$ transformed cell lines, in an attempt to obtain genetic complementation.

Significance: To enhance existing knowledge of tumor virology.

Proposed Course: Continuing project.

Project Description: Part II. Search for antibodies to SV$_{40}$ virus and T-antigen in children immunized with SV$_{40}$ contaminated poliomyelitis vaccines.

This is an extension of a study reported last year to determine the possible persistence of SV$_{40}$ infection in some vaccinees.

Major Findings: Sera of 28 school children who received the field trial vaccine in Grand Rapids, Michigan during 1954 were made available for this study by Dr. Gordon C. Brown. The vaccines used is this area were prepared by the Maitland culture procedure. Sera of each vaccinee included pre- and post-primary specimens (1954), pre- and post-booster (1955) and annual samples obtained for the next 5 years.

Only 4 of 28 vaccinees developed low titered neutralizing antibodies to SV$_{40}$ which disappeared within 1 year of the booster. None of the sera contained

detectable antibodies to the tumor antigen. These negative findings were not surprising since Maitland culture vaccines are known to contain only trace amounts of SV_{40}.

Significance: Information on the possible oncogenicity of SV_{40} administered parenterally to humans is of importance to the mission of DBS.

Proposed Course: Project discontinued.

Honors and Awards: None

Publications:

Paul Gerber: Patterns of Antibodies to SV_{40} in Children Following the Last Booster with Inactivated Poliomyelitis Vaccines. Proc. Soc. Exper. Biol. Med. 125: 1285-1287, 1967.

Serial No. DBS 126
1. Laboratory of Viral Immunology
2. Section on Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigations concerning combined, live virus vaccine.

Previous Serial Number: DBS 126

Principal Investigator: Harry M. Meyer, Jr., M.D.

Other Investigators: Hope E. Hopps, M.S.
Lewellys F. Barker, M.D.
Barbara C. Meyer, M.S.
Robert D. Douglas

Cooperating Units: None

Project Description: Investigations with vaccinia, measles and yellow fever viruses.

During the past year, as an outgrowth of research initiated by our group in 1962, a measles-smallpox vaccine became commercially available. In anticipation of the license of this material studies were conducted to develop tests appropriate for the potency assay of each of these virus components in the presence of the other. The titer of vaccinia virus could be determined with accuracy by either cytopathic effect or plaque production methods using BS-C-1, primary rhesus monkey kidney or primary rabbit kidney cells, as well as chorioallantoic membranes of 11 day chick embryos. The measles virus titer could be determined by the use of antiserum and appropriate chemotherapy to remove vaccinia virus from the BS-C-1 cell cultures used in the measles potency assay.

Our current effort has been directed toward studies with vaccinia. Interest, here, is in the development of a combined vaccine with a more modified vaccinia virus; see Project 47, p. 175.

Serial No. DBS 128
1. Laboratory of Viral Immunology
2. Section on Viral Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on infectious viral nucleic acid.

Previous Serial No. Same

Principal Investigator: Marvin S. Amstey, M. D.

Other Investigators: Bruce Mann, B.S.

Cooperating Units: None

(Project completed; no further studies planned).

Serial No. DBS 129

1. Laboratory of Viral Immunology
2. Section on Viral Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Demonstration of a New Congenital Anomaly in the Rabbit.

Previous Serial No. Same

Principal Investigator: Marvin S. Amstey, M.D.

Other Investigators: None

(Project completed; no further studies planned).

Serial No. DBS 130
1. Laboratory of Viral Immunology
2. Section on Viral Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on ferritin-conjugated antibodies.

Previous Serial No. Same

Principal Investigator: Marvin S. Amstey, M.D.

Other Investigators: Bruce Mann, B.S.

(Project completed; no further studies planned).

Adjuncts to OD

Contracts

Investigators

Publications

Subject In

LVR

Annual Report of the
Laboratory of Virology and Rickettsiology
Division of Biologics Standards
July 1, 1967 through June 30, 1968

It is our contention that a large laboratory, such as this, should comprise several closely integrated but nonetheless independently functioning groups, each under a responsible investigator. In today's and, more importantly still, tomorrow's climate of vaccine making and regulations, appropriate research and control units of DBS should expand their concern to the pertinent fundamental aspects of virology and rickettsiology. Professional and technical competencies, capable of coping with problems that come up in relation to human vaccines, should be developed and important contemporary techniques must be available.

This is the reason why in addition to developing an active Section on Immunoserology, the research program of the old Basic Virology and Rickettsiology Section has been completely modified. A new program has been underway concerned with the pathogenesis of slow, latent and chronic virus infections (which may be involved as vaccine contaminants or eventual vaccine candidates), with quantitative approach to the problems of potency of certain vaccines, and with the understanding of the basic problems confronting the science of immunology today. This has necessitated introduction of several tools previously not available in this Laboratory, including electron microscopy, immunofluorescence, radioisotope and certain biochemical techniques. The approach has been reflected in the functional reorganization of the Laboratory, focused on the development of new research ideas and skills by "old" and newly recruited professional staff.

HIGHLIGHTS OF INDIVIDUAL PROJECT REPORTS

Influenza vaccines. During the year the influenza control program was re-examined by the newly organized Respirovirus Unit. Several traditionally weak areas were defined, especially in the performance of technical procedures of CCA determination and mouse potency tests. These procedures have now been standardized and a collaborative study between DBS and manufacturers' laboratories employing them is in progress. Two large studies on human volunteers inoculated with influenza vaccines of current virus strain composition were performed and the results are being analyzed to compare the immune responses elicited in man and mouse.

Yellow fever vaccine. The standard yellow fever vaccine seed has been purified by simple membrane filtration. The resulting primary and secondary vaccine virus seeds were shown

to be indistinguishable in animal experiments from the standard RIF-virus-contaminated yellow fever vaccine. Human volunteer trials are under way. In the course of these studies, a plaque neutralization test was developed; it seems to have many practical and theoretical advantages over the standard yellow fever mouse neutralization test.

Smallpox vaccine. It has been shown that electron microscopic counting of vaccinia virus particles extracted from smallpox vaccines may be useful in assaying the vaccine potency. In the course of this study, reactivation of heated poxvirus, using UV-inactivated virus as the reactivating virus, has been accomplished--an observation of considerable importance to the understanding of the biology of poxviruses.

Egg vaccines. Production of egg vaccines has been always complicated by the necessity of surveying the avian flocks for the potentially troublesome microbial infections. This involves bleeding the hard-to-capture birds, and examining the serum using a variety of serological procedures, depending on the antigen. Some of these procedures are cumbersome, relatively insensitive or non-specific. By combining the methodology of indirect immunofluorescence with the use of egg yolk extract (in place of bird serum), a simple, reliable, and inexpensive procedure has been worked out. It seems to be useful in detecting avian antibody against major groups of microbial antigens, and has distinct practical advantages for certain antibody tests.

Rickettsial vaccines. Several problems of practical and fundamental importance in relation to rickettsial vaccines are being investigated. Work continues on the definition of usefulness of complement fixation test in assaying the potency of typhus vaccines. Fundamental studies on the relationships among rickettsiae and on their relationship with host cells are in progress, employing biochemical and biological procedures, including DNA base ratio determination and homology trials. In the experiments using Rickettsia quintana as a model rickettsia, important nutritional requirements of the organism in cell culture are being defined.

Hemorrhagic fevers. The importance of our continuing studies on simian hemorrhagic fever has been underscored by the isolation of a virus from rhesus monkeys with hemorrhagic disease at the Sukhumi, USSR, primate colony. The Sukhumi virus was found to be indistinguishable from the Bethesda virus we had isolated and reported on last year. Recently another outbreak, apparently due to the same agent, occurred at the National Center for Primate Biology (Davis, California); on request we are assisting with its investigation. Other collaborative studies on the pathogenesis of hemorrhagic fevers suggest that, in common with certain other viral infections, the underlying mechanism may lie in the now

well defined clinical-pathological syndrome of "consumptive coagulopathy". This suggests that paradoxically anticoagulants might be useful in the treatment of viral hemorrhagic fevers.

Slow, chronic and latent infections and diseases. A new class of unusual agents causing remarkable diseases in man (kuru) and animals (scrapie, visna) has been defined. Because of the potentially great importance of some of these agents as possible contaminants of biologics products, an important project was initiated (coordinated with another new project on the auto-immune mechanisms that become involved through the action of live or inactivated virus antigens).

Cell cultures. A many faceted program of research and development in connection with the production of cell cultures for the Division is actively pursued. As a result, many practical improvements have been introduced into routine cultivation of cells from usual and unusual sources. Increasingly greater attention is being focused on the biology of cultured cells, including certain immunologic aspects.

Mollusk extracts. This long term project continues to accumulate evidence of antiviral and antitumor activity of extracts derived from marine mollusks, especially clams and their livers. The possibility that the active principle may be related to the enzyme l-asparaginase is currently under investigation.

CONTRACT ACTIVITIES

The only current contract is with Melpar, Inc., which furnishes electron microscope facilities for the study of vaccinia and other viruses (see Individual Project Report #124). This contract has recently been renewed for one year to continue the research in progress until our own instrument is finally installed.

SERVICE AND CONTROL FUNCTIONS

The Tissue Culture Section continues to spend most of its effort on the purely service function of providing the entire Division with cell cultures of research quality also suitable for control uses. Additional time was spent on safety testing of inactivated virus vaccines prepared in cell cultures. Much effort is made to improve the Section's service functions and to insure adequate cell culture production. All of the DBS continuous cell line stock has been replaced with certified, PPLO-free cells and the cell bank is operating at an efficiency which should allow almost immediate return to full scale production in case of accident or contamination.

During the calendar year 1967, the Section had prepared--395,833 cell culture tubes, 93,118 2-oz. bottles, 10,841 milk dilution bottles and 2,503 32-oz. bottles of primary cells and

cell lines (14) routinely carried by the Section. In addition, the Section prepared 8,710 ml of cell suspension and 4,870 liters of media. Increasing numbers of lots of primary cells of rabbit, hamster, and marmoset kidney and of mouse and hamster embryo, human thyroid and clam liver were processed on request from interested investigators. Production records of primary and serial cell cultures are summarized in Tables 1, 2 and 3.

The Avian Leukosis Unit performed 300 RIF tests during the reporting period, of which 10 were on 8 live measles vaccines, 8 on mumps vaccines or normal CETC fluids on which mumps vaccines were produced, 5 on rubella vaccines or normal CETC fluids on which rubella vaccines were produced, 11 on RIF-free yellow fever primary and secondary seeds and vaccines, and 266 as "egg checks" on SPAFAS and Kimber Farms eggs.

The new Respirovirus Unit received 68 influenza virus vaccines which were tested as shown in Table 4 in parallel by CCA test and 3 different neutralization test techniques.

Table 1
Report for Calendar Year 1967
CELL YIELD FROM PROCESSED TISSUES

Type of Tissue	Grams of Tissue Processed	Cells per Gram of Tissue
Kidney		
Monkey, rhesus	851	131×10^6
Monkey, cercopithecus	980	146×10^6
Rabbit	681	73×10^6
Marmoset	6	87×10^6
Embryo		
Chick	1,049	99×10^6
Mouse	10	56×10^6
Hamster	965	52×10^6
Other		
Human Thyroid	3	10×10^6
Clam Liver	35	123×10^6

Table 2
Report for Calendar Year 1967
NUMBER OF PRIMARY CELL CULTURES ISSUED

Type of Culture	Tubes	Bottles		
		2 oz.	MDB*	32 oz.
Kidney				
Monkey, rhesus	50,091	7,602	-	197
Monkey, cercopithecus	153,165	8,583	1,013	136
Rabbit	31,893	2,454	296	112
Marmoset	50	-	-	12
Embryo				
Chick	-	5,544	-	20
Mouse	100	-	-	-
Hamster	50	3,332	-	9
Other				
Human Thryoid	20	-	-	-
Clam Liver (suspension only)				

*MDB-milk dilution bottles.

Table 3
Report for Calendar Year 1967
NUMBER OF SERIAL CELL CULTURES PREPARED FOR ISSUE IN TUBES AND BOTTLES FROM 23,187 BOTTLES (32 oz.) MAINTAINED AS CELL STOCK

Type of Cultures	Tubes	Bottles		
		2 oz.	MDB[a/]	32 oz.
Human				
WI-38 (Hayflick)	41,096	1,849	-	61
HEp-2 (PD & Co)	559	443	-	295
Human amnion (Fogh & Lund)	30	-	-	104
HeLa (ATCC)	175	735	-	202
KB (ATCC)	25	8	-	55
Chang's Liver (Chang)	818	794	353	-
Animal				
RK-13 (Beale)	58,340	13,300	-	219
MA-104 (MBA)	37,222	44,325	-	464
BS-C-1 (Hopps)	13,699	3,225	9,039	195
BS-C-1 (Hopps/Merck)	500	-	-	-
L (NCTC 2071)	7,130	156	-	2
BHK-21 (Macpherson & Stoker)	870	740	140	374
LMK (Hull)	-	128	-	16
MDCK (Madden & Davis)	-	-	-	30

Table 4
Formalin-Inactivated Influenza Virus Vaccines Tested in the Respirovirus Unit from July 1, 1967 through April 30, 1968

Influenza Vaccines Received	Number received	Number Tested by Indicated Technique			
		*CCA	Mouse Neut.	Tissue Culture Neut.	Egg Neut.
Civilian bivalent	42	33	27	27	1
Civilian polyvalent	15	14	10	11	1
Military	11	4	1	1	0
TOTAL	68	51	38	39	2

*CCA = Chicken red blood cell agglutination test.

CONTRACT NARRATIVES
DIVISION OF BIOLOGICS STANDARDS
July 1, 1967 through June 30, 1967

MELPAR, INC. (PH43-67-1105)

Title: Electron Microscope Services

Contractor's Project Director: Robert Hermann

Project Officer (DBS): Ruth Dunlap, Ph. D.

Objectives: To incorporate the electron microscope into the studies of vaccinia and other viruses.

Methods Employed: Sophisticated use of the electron microscope.

Major Findings: The electron microscope facilities at Melpar have been utilized regularly, enabling data obtained by this source to be incorporated into all investigations concerning vaccinia virus. Further information on the specific usage of this data is provided in Individual Project Report #124. The microscope has been utilized by other LVR investigators to visualize aspects of their work.

Significance to Bio-Medical Research and the Program of DBS: Electron microscopic examination of smallpox vaccine provides knowledge of the actual number of vaccinia virus particles present and their degree of dispersion. This quantitation supplies the one base of reference to correlate all existing titration methods. In general, visualization of virus particles contributes invaluable information concerning infectivity ratios, virus yields and morphology.

Proposed Course of Project: Contract has been extended for a period of 1 year or until such services can be supplied by DBS.

Date Contract Initiated: Contract was initiated May 22, 1967 for $14,560. It has been renewed for 1 year as of May 22, 1968, at no additional funding.

Current Annual Level: $14,560.

Serial No. 57
1. Virology & Rickettsiology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Office of the Chief

Previous Serial No.: Same

Principal Investigator: Alexis Shelokov, M.D.

Other Investigators: None

Cooperating Units: None

Man Years
Total: 4.6
Professional: 0.6
Other: 4.0

Project Description:

Objectives: Administration, planning and coordination of research and control activities of the Laboratory's sections.

The project furnishes supervisory and administrative services to all research projects and biologics control activies in the laboratory as follows: (a) Over-all planning of intramural research, collaborative (contract) and control functions and their coordination among and within the several Sections of the Laboratory, (b) supervision over personnel, maintenance, travel and correspondence, (c) supervision over requisitioning and maintenance of equipment and supplies, (d)reviewing, editing and revising scientific and technical reports and manuscripts, (e) preparation of administrative reports, exhibits and other publication materials, (f) participating with other Laboratory Chiefs in staff functions for Office of the Director, including staff committees which ensure smooth operation of the Division in its many types of activity, (g) official liaison and communications with the manufacturers of viral vaccines and other biologics, (h) consultation services to individuals, academic institutions and government agencies, including liaison activities with other branches of the Service, foreign governments and international agencies, (i) maintenance of reference library and reprint files.

Honors and Awards:

Member, Scientific Advisory Board, Gorgas Memorial Institute, Washington, D. C. and Gorgas Memorial Laboratory, Panama (re-appointed 1967-68).

Invited participant, XIII Scientific Session, Institute of Poliomyelitis and Virus Encephalitides, Academy of Medical Sciences, USSR, June 27-30, 1967.

Invited participant in Working Conference on Smallpox, sponsored by U.S.-Japan Cooperative Medical Science Program, Office of International Research, National Institutes of Health, Honolulu, Hawaii, December 6-8, 1967.

Associate Member, Commission on Viral Infections, Armed Forces Epidemiological Board, Department of the Army, Washington,D.C. (appointed 1967-1969).

Invited participant in Workshop on Epidemiological Surveys, sponsored by National Academy of Sciences, National Research Council, Washington, D. C., January 8-10, 1968.

Member, Virology and Rickettsiology Study Section, Division of Research Grants, National Institutes of Health (appointed 1968-1971).

Member, N.I.H. Board of Employee Awards (appointed June 28,1967).

Invited participant, Workshop on Virus Diseases Which Impede Colonization of Nonhuman Primates, National Center for Primate Biology, University of California, Davis, California, May 22-24, 1968.

Serial No. 139
1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on autoimmune reactions related to viral and adjuvant sensitization

Previous Serial No: None

Principal Investigator: Paul Albrecht, M.D.

Other Investigators: Alexis Shelokov, M.D.
Ronald Clark, Ph. D.
Ruth Kirschstein, M.D.

Cooperating Units: Laboratory of Pathology, DBS

Man Years:

Total: 1.4
Professional: 0.4
Other: 1.0

Project Description:

Objectives: (1) Select a suitable experimental model permitting variations and effective measurement of humoral and cellular immunity to viruses considered, e.g., herpes simplex, measles, representatives of arboviruses group B and possibly rabies; (2) Evaluate the effect of repeated injection of adjuvant alone, adjuvant plus viral antigen, and virus alone on tissue susceptibility and tissue vulnerability of the sensitized host.

Methods Employed: Conventional microbiological and serological techniques, and testing of cellular sensitization by radioisotope techniques.

Major Findings: Fourteen inbred and 3 randomly bred mouse strains and 3 inbred rat strains were injected with homologous or heterologous brain tissue in adjuvants. The individual strains deferred markedly in the extent to which they reacted with CNS involvement. Histological examination and serological studies are underway to complete evaluation of susceptibility to autoimmune encephalitis.

Significance to Bio-Medical Research and the Program of DBS: It is obviously of importance to the mission of the Division to elucidate the autoimmune mechanisms which become involved by the action of live or inactivated virus antigen.

Honors and Awards: None.

Publications:

Albrecht, P.: Pathogenesis of neurotropic arbovirus infections. In Current Topics in Microbiology and Immunology. Vol. 43, Springer Verlag, Berlin-Heidelberg-New York, 1968.

Serial No. 140
1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on agents of chronic and degenerative diseases

Previous Serial Number: None

Principal Investigator: Paul Albrecht, M.D.

Other Investigators: Alexis Shelokov, M.D.
Richard A. Mason, D.V.M.
Ruth Kirschstein, M.D.

Cooperating Units: Laboratory of Pathology, DBS

Man Years: (The project was in operation ½ of fiscal year.)

Total: 1.4
Professional: 0.4
Other: 1.0

Project Description:

Objectives: A new class of agents has been defined, causing disease in man (kuru, spongiform encephalopathy, ALS?) and animals (scrapie, visna, maedi, NZB mouse agent) which show unusual properties, e.g. latency, long incubation period--months to years; protracted course of serious disease, pathological changes unlike those of acute viral disease.

Some of the agents are unusually resistant to heat, formalin, radiation and pH. They seem to be ubiquitous with the manifestations of disease dependent on the genetic composition of the host and circumstances of transmission.

As a rule, their cultivation and identification still poses a formidable problem. To monitor confidently for the presence of "slow virus" agents in biological materials, better assay systems than presently available must be developed.

Methods Employed: Physico-chemical techniques to determine biological properties; search for suitable animal hosts and tissue culture systems; detection of agents by interference, fluorescent antibody and radio-isotope techniques.

Major Findings: Mice were inoculated with increasing dilutions of the scrapie agent and their brain assayed by the fluorescent antibody technique

at monthly intervals. Definite lesions were first detectable 2-3 months after infection, while 5 months elapsed before clinical symptoms developed. Animals inoculated at higher dilutions are still under observation.

Other investigators have failed to detect antibodies to the scrapie agent. Apparently, because of this lack of humoral response, we were not able to develop a direct fluorescent antibody test for scrapie using infected mouse brain as antigen. Further work using immunlfluorescence is necessary.

Significance for Bio-Medical Research and the Program of the Division: Dealing with a new class of infectious agents and possible contaminants of biological products, the studies are intended to give competence to DBS workers in this field and join in developing monitoring techniques and measures.

Proposed Course of Project: The project will continue at the present level.

Honors and Awards: None.

Publications: None.

Serial No. 75

1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on arthropod-borne viruses (arboviruses)

Previous Serial Number: Same

Principal Investigators: Nicola M. Tauraso, M.D.
Alexis Shelokov, M.D.

Other Investigators: Frank A. Pedreira, M.D.

Cooperating Units: Institute of Child Health and Human Development

Man Years:

Total: .8
Professional: .1
Other: .7

Project Description:

Objectives: (1) To maintain a laboratory facility with competency in the techniques of animal work, cell culture and serology for the study of arboviruses; (2) to maintain a reference collection of arboviruses, including those of importance as vaccine candidates, and (3) to conduct studies on select arboviruses, especially those of importance as potential vaccine candidates.

Major Findings: The arbovirus facility now has the professional and technical competency capable of performing studies on the arboviruses, utilizing the techniques of animal inoculation, cell culture and serology.

The working collection of viral and antibody reagents has expanded considerably. Present reagents representing a number of virus strains for most of the major and minor arbovirus groups are available and are being utilized for routine and special studies. During this year studies were continued with emphasis on simian hemorrhagic fever, American hemorrhagic fever and yellow fever vaccine viruses (reported separately under project Nos. 118 and 121 respectively.

Viruses of the California group are becoming increasingly significant causes of encephalitis in the United States. During our studies on the biology and immunology of the Bunyamwera and California virus groups we observed that Guaroa virus suspensions contained two different plaquing

populations. A possibility has been considered that Guaroa virus preparations contain a mixture of viruses and that this mixture might explain the fact that Guaroa virus is serologically related to two heretofore different groups, namely Bunyamwera and California. The prototype CoH 35211 strain of Guaroa virus contained two different plaque populations when French or Mann agarose were used for solidifying the overlay medium. The pl 1L (large) and pl 2S (small) plaque variants were similar with respect to pathogenicity for suckling mice (SM), titer achieved in SM brains after intracranial inoculation and time of appearance of plaques under agarose overlay. The "wild" CoH 35211 strain and the two plaque variants were indistinguishable by complement fixation and plaque neutralization (PN) tests. However, the two plaque variants differed with respect to plaque size and sensitivity to the inhibitory effects of sulphated agar polysaccharides. The only different serological relationship was a "one-way" cross-reaction between pl 2S antibody and California encephalitis and Tahyna viruses by PN test.

Significance to Bio-Medical Research and the Program of DBS: Many of the continental states, as well as Hawaii, Alaska and Puerto Rico, possess the ecological prerequisites of potential vectors and reservoirs necessary for initiation of arbovirus epidemics. Vaccines against arboviruses of importance to the world--the U.S. personnel and travelers abroad--are being developed. Some may soon come within the purview of the Division of Biologics Standards. Further knowledge of the biochemical and biophysical properties is needed in order to appreciate more the unique features of arboviruses and before these viruses can be handled as biologic products.

Proposed Course of the Project: Research on selected arboviruses will continue.

Honors and Awards: None.

Publications:

Tauraso, N. M., and Shelokov, A.: Arboviruses--a problem in classification. Arch. ges. Virusforsch. 22: 273-279, 1967.

Shelokov, A. and Peralta, P. H.: Vesicular stomatitis virus, Indiana type an arbovirus infection of tropical sandflies and humans? Amer. J. Epidemiol. 88: 149-157, 1967.

Serial No. 118
1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on hemorrhagic fever viruses

Previous Serial Number: Same

Principal Investigator: Nicola M. Tauraso, M.D.

Other Investigators: Alexis Shelokov, M.D.
Anton M. Allen, D.V.M.
Amos E. Palmer, D.V.M.
Calvin G. Aulisio, M.S.
Lewis H. Dennis, M.D.

Cooperating Units: Comparative Pathology Section, LAB, DRS
Laboratory of Pathology, DBS
Department of Hematology, WRAIR

Man Years:

Total: 1.7
Professional: .4
Other: 1.3

Project Description:

Objectives:

A. (1) To maintain a facility for working with the important viruses causing hemorrhagic fever in the western hemisphere with emphasis on the immunological vaccine aspects; (2) to maintain practicable cell culture and animal host systems and serological tools for working with this group of viruses; (3) to study the immunological relationships of these viruses using in vitro serological tests and in vivo animal protection tests.

B. (1) To study the pathophysiology of hemorrhagic fever using monkeys infected with simian hemorrhagic fever (SHF) virus as a model; (2) to attempt to isolate a virus from specimens obtained from monkeys which died of hemorrhagic fever at the Sukhumi Institute of Experimental Pathology and Therapy, USSR, in July, 1964 and to determine the relationship of that disease with the hemorrhagic fever which occurred at the Primate Quarantine Unit of LAB in the Fall of 1964.

Methods Employed:

A. Studies were performed on 2 members of the Tacaribe group of American hemorrhagic fever viruses. Adult guinea pigs were immunized with

live Tacaribe virus (not known to be pathogenic for any adult animal) and later were challenged with Junin virus (Argentinian hemorrhagic fever virus) to which they are normally quite susceptible. At various intervals following immunization and following challenge, blood samples were obtained from immunized and control animals in order to determine the extent of viremia, the defects in coagulopathy, and antibody production.

B. Utilizing the broad resources available to the Arboviruses Facility of LVR, an attempt was made to establish the etiology of a simian hemorrhagic fever occurring at the Sukhumi Institute of Experimental Pathology and Therapy, USSR. The techniques of animal and egg inoculation and of cell culture were employed in an effort to isolate an infectious agent from specimens obtained from sick and moribund monkeys. Studies to determine the extent of the coagulation defects in SHF virus infected monkeys were performed.

Major Findings:

A. One of the investigators (Dr. Dennis) recently demonstrated that the marked thrombocytopenia seen in the arbovirus infections, Korean hemorrhagic fever (hemorrhagic nephroso-nephritis) and yellow fever, was caused by disseminated intravascular coagulation and associated with multiple defects in the first and second stages of coagulation, hypofibrinogenemia and accumulation of fibrinogen degradation products. Our studies described in this report demonstrated a similar phenomenon in guinea pigs infected with Junin virus. Coagulation abnormalities occurred approximately 7 days after viral inoculation and prior to clinical illness; they increased in severity until the animal's death with generalized hemorrhage. The coagulopathy is characterized by prolonged prothrombin time and partial thromboplastin time, reflecting deficiencies in factors, V, VII, VIII, IX, X, XI and XII; a prolonged prothrombin time despite normal fibrinogen levels, suggesting an accumulation of fibrinogen degradation products; lack of compensatory fibrinolytic activity as reflected by prolonged euglobulin lysis time; as well as marked thrombocytopenia and leukopenia. The coagulopathy picture is similar in monkeys infected with SHF virus. These antemortem studies demonstrating accelerated intravascular coagulation, together with post-mortem histologic findings of thrombosis and hemorrhage, suggest that anticoagulants might be important in the treatment of hemorrhagic fevers.

B. Serum samples were obtained from monkeys involved in the Sukhumi epizootic of simian hemorrhagic fever. Two sera were selected and inoculated intramuscularly into rhesus monkeys housed in a specially constructed negative pressure isolator. Both monkeys developed a disease indistinguishable from that seen during the NIH epizootic. From serum obtained from each of these 2 monkeys when they were moribund, a viral agent, which we have designated the Sukhumi strain of SHF virus, was isolated in tube cultures of

MA-104 embryonic rhesus monkey kidney cell line. After several passages in cell culture, the virus caused typical clinical disease when inoculated into healthy rhesus monkeys. The Sukhumi strain was serologically indistinguishable from the virus strains obtained from the NIH epizootic.

Significance to Bio-Medical Research and the Program of DBS:

A. Concerning the studies on the American hemorrhagic fever viruses, there is no doubt that viral hemorrhagic fevers have become a major public health concern in the Americas. So far attempts to provide a safe and adequate vaccine against the Argentinian disease have been unsuccessful. During the most recent epidemics of Bolivian hemorrhagic fever much consideration was given to the possibility of immunization as an eventual control measure. It would be in keeping with the mission of this Division to develop and maintain the compentency able to cope with the problems of the evaluating potential vaccine candidates. The ante-mortem studies demonstrating accelerated intravascular coagulation in the hemorrhagic fevers suggest that anticoagulant therapy might play a significant part in reducing rates of morbidity and mortality in these diseases for which their is no specific treatment.

B. Since primates are needed by the Division in carrying out the required safety tests on some vaccines, it is only logical that the arbovirus facility of LVR maintain a competency able to cope with certain aspects of endogenous viral diseases of monkeys.

Proposed Course of the Project:

Studies on the American hemorrhagic fever and simian hemorrhagic fever viruses will be continued.

Honors and Awards: None

Publications:

Palmer, A. E., Allen, A. M., Tauraso, N. M., and Shelokov, A.: Simian hemorrhagic fever. I. Clinical and epizootiological aspects of an outbreak of quarantined monkeys. Am. J. Trop. Med. Hyg., 17: 404-412, 1968.

Allen, A. M., Palmer, A. E., Tauraso, N. M., and Shelokov, A.: Simian hemorrhagic fever. II. Studies in pathology, Am. J. Trop. Med. Hyg., 17: 413-421, 1968.

Tauraso, N. M., Shelokov, A., Palmer, A. E., and Allen, A. M.: Simian hemorrhagic fever. III. Isolation and characterization of a viral agent. Am. J. Trop. Med. Hyg., 17: 422-431, 1968.

Tauraso, N. M., Shelokov, A., Allen, A. M., Palmer, A. E., and Aulisio, C. G.: Simian hemorrhagic fever: isolation and identification of the virus causing an epizootic in Sukhumi, USSR. Nature (in press) 1968.

Serial No. 121
1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on Yellow Fever Vaccine

Previous Serial Number: Same

Principal Investigators: Nicola M. Tauraso, M.D.
Sheldon L. Spector, M.D.
Alexis Shelokov, M.D.

Other Investigators: William G. Jahnes
Ruth L. Kirschstein, M.D.
Edward B. Seligmann, Jr., P. D.

Cooperating Units: Laboratory of Control Activities, DBS
Laboratory of Pathology, DBS

Man Years:
Total: 3.1
Professional: 1.4
Other: 1.7

Project Description:

Objectives: (1) To purify the current Yellow Fever Vaccine by removing the contaminating avian leukosis virus; (2) to evaluate the neurovirulence of the new candidate in monkeys; (3) to study the immunogenicity of the purified vaccine virus candidate in monkeys and man; and (4) to attempt to select by plaque-purification a strain with less neurovirulence than the current vaccine strain.

Methods Employed: Utilizing the broad resources available to the Arbovirus, Immunoserology, and Avian Leukosis Units of LVR an attempt is being made to investigate the various aspects of this project by common laboratory techniques.

Major Findings: The DBS yellow fever project was formally established in April 1966 by the appointment of a project officer (Dr. Tauraso) and two assistant project officers (Dr. Seligmann, LCA, and Mr. Jahnes). Several meetings of interested laboratory chiefs and other DBS staff were held, and a coordinated research program was established and is currently well underway. Previous to the establishment of this official yellow fever project we realized that LVR offered an unusual combination of competency, facilities and interest with regard to arboviruses and avian leukosis viruses, which to our knowledge is unique in the world. This

placed us in a position to undertake a comprehensive re-evaluation of the standard Yellow Fever Vaccine known to be contaminated with the RIF agent and to attempt development of a RIF-free attenuated yellow fever virus seed. The decision was made to execute this project as a joint intramural effort and to augment particular specialized needs by utilizing the contract mechanism.

The first objective has been achieved. The size differential between yellow fever virus (less than 50 mμ) and RIF virus (approximately 100 mμ) allowed us to purify yellow fever virus by simple Millipore filtration. Using an early passage (229) of Yellow Fever Vaccine virus as seed, a pool was prepared in RIF-free eggs containing yellow fever virus and no detectable avian leukosis viruses. This primary seed, as well as subsequent secondary seeds and vaccines were prepared and confirmed as being free from avian leukosis virus by RIF, COFAL, and FAB tests.

The second objective has also been accomplished. The new avian leukosis virus-free and the current RIF virus-contaminated primary and secondary vaccine seeds were indistinguishable by potency and monkey neurovirulence tests.

The third objective is well underway. Serological tests performed thus far show that RIF virus free vaccines are as immunogenic as the contaminated vaccines when administered to rhesus monkeys. Plans have been made to immunize human volunteers; these studies will soon be performed.

As part of the Yellow Fever Vaccine studies, we have developed a convenient plaque neutralization (PN) test using the MA-104 embryonic rhesus monkey kidney cell line and a single agar-overlay procedure. When compared to mouse neutralization (MN) tests using suckling and weanling mice inoculated by the intracranial route, the PN test was: the most sensitive for measuring neutralizing antibody, less variable, less costly and quicker (5-6 days vs 21 days for the PN and MN tests respectively) in achieving results.

Significance to Bio-Medical Research and the Program of DBS: A re-evaluation of old vaccines using more up-to-date methods is one of the responsibilities of the Division. The development of a Yellow Fever Vaccine free of contaminating avian leukosis virus is obviously advantageous to humans receiving this vaccine.

Proposed Course of Project: These studies are being continued as outlined.

Honors and Awards: None.

Publications:

Tauraso, N. M., Spector, S., Jahnes, W. G., and Shelokov, A.: Yellow fever virus vaccine. I. Development of a vaccine seed free from contaminating avian leukosis viruses. Proc. Soc. Exp. Biol. & Med. In press. (1968).

Serial No. 122

1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on respiratory viruses. I. Influenza virus vaccines

Previous Serial Number: Same

Principal Investigators: Jose G. Canchola, M.D.
Nicola M. Tauraso, M.D.

Other Investigators: Frank A. Pedreira, M.D.
Alexis Shelokov, M.D.
Ruth C. Dunlap, Ph. D.

Cooperating Units: Laboratory of Control Activities, DBS
Employee Health Services, NIH
Respiratory Infections Unit, NCDC

Man Years:

Total: 8.0
Professional: 2.2
Other: 5.8

Project Description:

Objectives: (1) To maintain a competent Respirovirus Unit for the study of influenza viruses and other viral agents etiologically associated with respiratory tract infections of man; (2) to maintain a reference collection of infectious viral antigens, and antibody reagents of influenza viruses and other respiroviruses including those of potential importance as vaccine constituents; (3) to review and analyze manufacturers' protocols and to perform immunogenic potency tests on current influenza virus vaccines; (4) to improve serologic techniques for immunogenic potency testing of influenza virus vaccines; (5) to compare reproducibility of serologic tests for immunogenic potency of current influenza virus vaccines; and (6) to conduct immunogenic studies with influenza viruses of epidemiologic importance in the United States.

Methods Employed: Standard virologic and serologic methods are employed in these studies.

Major Findings: (1) The serologic tests (egg, mouse and tissue culture neutralization) used for vaccine potency evaluation and the CCA technique have been standardized. (2) Specific immune guinea pig sera have been pre-

pared against representative influenza virus strains and are available; these sera are undergoing intensive testing in the laboratory. (3) In the Fall of 1967, two major studies were carried out in adult human volunteers immunized with current formalin-inactivated influenza virus vaccines. These studies were designed to evaluate the immunogenic capacity of these vaccines in man and to compare these findings with the immune response elicited in experimental laboratory animals. At present, the serologic tests are still in the process of completion and analysis. Virus isolation in tissue culture is being attempted with specimens collected from vaccinated individuals with febrile respiratory tract illnesses. (4) Efforts to compare the egg, mouse and tissue culture neutralization tests for measuring antibody produced in the mouse potency tests were inconclusive, probably because of the variability of the reagents utilized. A large collaborative study is now underway in collaboration with influenza vaccine manufacturers employing reagents which have been standardized. Hopefully, this will enable us to choose the most accurate and reproducible method for measuring antibody in the mouse potency test.

Significance to Bio-Medical Research and the Program of DBS: DBS is responsible for the control and potency testing of current influenza virus vaccines. Sustained and comprehensive scientific research, as performed by the Respirovirus Unit, is essential for the improvement and development of antigenically competent influenza virus vaccines.

Proposed Course of Project: Studies on influenza viruses and influenza vaccines will continue.

Honors and Awards: None.

Publications: None.

Serial No. 123
1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on adenoviruses

Previous Serial Number: Same

Principal Investigators: Frank A. Pedreira, M.D.
Nicola M. Tauraso, M.D.

Other Investigators: Alexis Shelokov, M.D.
Amos E. Palmer, D.V.M.
Ruth L. Kirschstein, M.D.

Cooperating Units: Institute of Child Health and Human Development
Laboratory of Pathology, DBS

Man Years:

Total: .6
Professional: .3
Other: .3

Project Description:

Objectives: In an attempt to establish a laboratory model for further evaluation of the live oral adenovirus vaccine, infectivity and transmission studies were instituted in rhesus monkeys. The purposes of the study are: (1) to attempt to infect monkeys inoculated intramuscularly, intravenously; (2) to study the virus transmission to uninoculated cage-mate controls; (3) to determine the antibody response of inoculated and uninoculated animals and (4) to test for neurovirulence in monkeys inoculated intrathalamically and intraspinally.

Methods Employed: Standard virological and serological techniques were employed. Monkeys were randomly segregated into three groups and housed in pairs. One animal from each pair served as an uninoculated cage-mate control. The 3 groups were inoculated with prototype adenovirus type 4 via the intramuscular, intravenous, and intranasal routes respectively. Blood specimens, throat and rectal swabs were collected from each animal prior to inoculation. Subsequent specimens were collected over a 3-week period and were processed for viral isolation and serology.

Major Findings: (1) The pre-inoculation monkey sera were free from neutralizing antibody to adenovirus type 4. (2) Adenovirus isolation attempts on pre-inoculation specimens were negative for all groups. (3) From 162

post-inoculation specimens collected over a 14-day period from the inoculated monkeys, 9 viruses displaying typical adenovirus CPE were obtained. Five isolates were identified as adenovirus type 4 by neutralization. Three isolates were recovered from 2 of the monkeys inoculated intravenously and 2 from animals inoculated intranasally. Four of the 5 isolates were from anal swab specimens, the fifth from a buffy coat specimen. All of the isolates were identified as adenoviruses by CF tests; no attempt was made to type the isolates which were not type 4 adenovirus. Three of 4 monkeys, from which adenovirus type 4 was recovered, developed 4-fold or greater neutralizing antibody titers. All three animals also developed CF antibodies during the same time period. (4) From 216 specimens collected from the cage-mate controls over a 21-day period, 3 isolates with typical adenovirus CPE were recovered from anal swab specimens obtained from 2 of the 9 cage-mate controls. These isolates could not be identified as type 4 adenovirus by neutralization tests but did fix complement in the presence of type 4 antiserum. (5) Four isolates were obtained from anal swabs of a monkey inoculated intramuscularly. Although these isolates could not be identified as type 4 adenovirus by neutralization tests, this animal did develop a 64-fold rise in neutralizing antibody titer and 32-fold rise in CF antibody titer.

The above suggests that we might have infected rhesus monkeys with type 4 adenovirus by the intravenous, intranasal and possibly intramuscular routes. The large virus dose employed and the difficulties encountered indicate that these animals are not readily susceptible to infection and could not serve as useful models for vaccine testing. Communicability between inoculated and uninoculated cage-mates could not be demonstrated. The possibility exists that the isolates obtained represent a gradual mechanical excretion in the absence of infection and replication. Additional experience regarding infection and pathogenesis of human adenoviruses in rhesus monkeys is needed to resolve these questions.

Significance to Bio-Medical Research and the Program of DBS: Present investigation of infection and pathogenesis of human adenoviruses must be studied in humans since a suitable laboratory model has not been established. Recent attention has been focused on the development and testing of live oral adenovirus vaccine. It is consistent with the mission of the Division to perform studies on the infection and pathogenesis of these viruses in animals. If an animal model was established independent studies on the safety and potency of adenovirus vaccine could be readily conducted.

Proposed Course of Project: The studies described in this report have been completed. Any additional information which might develop from these studies will be reported as part of our general studies on respiratory viruses.

Honors and Awards: None.

Publications: None.

Serial No. 141

1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on endogenous viruses contaminating avian eggs. I. Duck hepatitis virus

Previous Serial Number: None.

Principal Investigators: Nicola M. Tauraso, M.D.

Other Investigators: George E. Coghill
Calvin G. Aulisio, M.S.

Cooperating Units: None

Man Years:

Total: .7
Professional: .3
Other: .4

Project Description:

Objectives: (1) To study systematically the properties of viruses known to contaminate eggs of avian species, and (2) to search for unknown agents endogenous to avian eggs and to develop tests for detecting these newer virus contaminants.

Major Findings: Recently, there has been renewed interest in the possible use of the developing duck embryo as a host for the production of virus vaccines. During the past year, we have concentrated our efforts in studying the properties of the attenuated vaccine strain of duck hepatitis virus. This strain grew well in 10-day-old embryonated chicken eggs and would grow to titers of 10^3 to 10^4 in trypsin dispersed primary chick embryo cell culture. Duck hepatitis virus was shown to have the following properties: RNA, <50 millimicrons in size, chloroform resistant, pH 3.0 resistant, trypsin resistant, relatively formalin sensitive, heat ($50^{\circ}C$) stable and no effect of 1M divalent cation upon heat stability.

Having these properties duck hepatitis virus would best classify as picornavirus.

Efforts to develop an immunofluorescence test for the detection of duck hepatitis virus antibody were unsuccessful.

Significance to Bio-Medical Research and the Program of DBS: When a new host is being considered for propagation of virus to be used for vaccines, it is imperative that knowledge be obtained concerning the endogenous agents of the new host. Although much is known concerning the virus contamination of developing chicken embryos, little is known about the agents which might contaminate developing duck embryos. Now that a live virus vaccine (i.e., mumps) produced in the developing duck embryo has been licensed for general use, a constant search for and knowledge of potential endogenous contaminating agents would ensure continued safety of the vaccine.

Proposed Course of the Project: Additional studies on duck hepatitis virus will be performed and attempts will be made to develop a serological test for detecting duck hepatitis antibody.

Honors and Awards: None.

Publications: None.

Serial No. 117

1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Avian leukosis viruses in relation to human vaccines

Previous Serial Number: Same

Principal Investigators: William G. Jahnes
Calvin G. Aulisio, M.S.

Other Investigators: Nicola M. Tauraso, M.D.
Alexis Shelokov, M.D.
Freddie Mitchell, B.S.
Henry Orr, Ph. D.

Cooperating Units: Laboratory Aids Branch, DRS
Laboratory of Infectious Diseases, NIAID

Man Years:

Total: 3.3
Professional: 2.1
Other: 1.2

Project Description:

Objectives: (1) To test live and inactivated vaccines prepared from chick embryo tissue or chick embryo cells in tissue culture for the presence of Resistance-Inducing-Factor (RIF); (2) to develop new and more sensitive procedures for detecting viruses of the avian leukosis complex; (3) to compare the relative effectiveness of the RIF, COFAL and fluorescent antibody (FAB) techniques for the detection and assay of several members of the avian leukosis virus complex; (4) to assist in the development of RIF-free vaccines prepared in chick embryos or chick embryo cell cultures.

Methods Employed: Standard and modified cell culture techniques, complement-fixation and FAB tests are employed.

The study outlined as objective No. 2 has been broadened to include the use of many different primary and continuous cell culture systems to screen for susceptibility to Rous sarcoma virus and other viruses of the avian leukosis complex.

Objective No. 3 is a cooperative effort of three groups of investigators within LVR. The Avian Leukosis Unit performs the RIF test according to the standard DBS procedure used in the testing of vaccines; the Serology Unit

performs the COFAL test (complement-fixation for avian leukosis); the FAB Unit performs the fluorescent antibody test. Antibody and antigen reagents are prepared and standardized for use in these studies; other techniques are utilized as required, such as cell culture, animal and egg inoculation with viruses, and tumor transplantation. These studies have also been expanded to include an evaluation of the NP test to detect viruses of the avian leukosis complex.

Major Findings: During this reporting period a total of 300 RIF tests were performed. Of these 10 were tests on 8 live measles vaccines which were found to be RIF and FAB test negative; 8 were tests on mumps vaccines or normal CETC fluids on which mumps vaccines were produced; 5 were tests on rubella vaccines or normal CETC fluids on which rubella vaccines were produced; 11 were tests on RIF-free yellow fever primary and secondary seeds and vaccines; 266 were miscellaneous tests on Kimber Farms and SPAFAS eggs used as back-up egg sources, positive and negative controls and virus titrations.

The production of RIF-free Yellow Fever Vaccine was completed using test methods described in a previous report (for further details see Project No. 121).

Due to the newness of the projects concerned with screening new cell culture systems for susceptibility to viruses of the avian leukosis complex and with evaluation of the NP test, no definitive results are available.

Significance to Bio-Medical Research and the Program of DBS: At present, the RIF test is routinely performed on vaccines for detecting viruses of the avian leukosis complex. The FAB test provides a rapid double check on vaccine testing and is a quick, easy method of continuous monitoring of RIF-free flocks. Because RIF-free chick embryo fibroblasts are required in the safety testing of vaccines, a RIF-free chicken flock must be maintained at NIH. The monitoring of this flock can be rapidly and easily achieved by using techniques to test eggs for maternal antibody by the indirect FAB technique. The development of more sensitive and more reliable procedures is of utmost importance to the Division's mission.

Proposed Course of Project: Work will continue on improving the present RIF, Fab, and COFAL tests and on developing simpler and more rapid procedures for the detection of the avian leukosis viruses. Studies on Marek's disease, a known important cause of avian leukosis, has been initiated with the hope of developing a satisfactory test system.

Honors and Awards:

1. Invited participant (Mr. Jahnes)--United States Department of Agriculture Working Converence on Avian Leukosis--RIF-free Vaccines, Washington, D. C., October 5, 1967 (Description of the RIF test procedure).

2. Invited participant (Mr. Aulisio)--United States Department of Agriculture Working Conference on Avian Leukosis--RIF-free Vaccines, Washington, D.C., October 5, 1967 (Description of the FAB test procedure).

3. Invited participant (Dr. Taurasco)--United States Department of Agriculture Working Conference on Avian Leukosis--RIF-free Vaccines, Washington, D. C., October 5, 1967 (Description of the COFAL test procedure and discussion of the relative sensitivity of the RIF, COFAL and FAB tests).

4. Sustained Superior Performance Award (Mr. Jahnes).

Publications: None

Serial No. 125

1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Egg yolk antibody as a tool to study avian microbial infections potentially contaminating human vaccines

Previous Serial Number: Same

Principal Investigator: Calvin G. Aulisio, M.S.

Other Investigators: Alexis Shelokov, M.D.

Cooperating Units: U.S. Department of Agriculture, Washington, D. C.

Man Years:

Total: 1.5
Professional: 0.8
Other: 0.7

Project Description:

Objectives: To study the nationwide prevalence of avian microbial infections present in eggs which may potentially contaminate human vaccines.

Methods Employed: Indirect immunofluorescence employing egg yolk in place of serum.

Major Findings: Work on this project was directed toward collection of information to assess the usefulness of substituting egg yolk antibody for serum antibody in the immunofluorescence test. Our findings suggest that the yolk immunofluorescence method can be advantageously used to study agents of all microbial groups (viruses, rickettsiae, bedsoniae, mycoplasmas, and bacteria).

We found 41 of 93 American chicken flocks with antibody to Twiehaus (RE) viral antigen. Although, Twiehaus virus is distinct from other avian leukosis viruses, it causes a similar clinical syndrome in infected chickens. It may be another potentially important contaminant in chicken eggs.

We found 90 of 93 American chicken flocks with antibody to subgroup A of avian leukosis viruses, or to subgroup B, or to both subgroups. The percentage of eggs with antibody to one or the other subgroups was about the

same. The capability of the immunofluorescence test to detect these different avian leukosis subgroups makes it an important adjunct to the RIF test in the safety testing of human viral vaccines of egg origin (see Serial No. 117).

Significance to Bio-Medical Research and the Program of DBS: Some licensed and experimental viral and rickettsial vaccines are prepared in chicken and other avian eggs. We now have a simple means to study the occurrence of various avian microbial infections in fowl. This may have broad biological and veterinary significance, besides providing the vaccine manufacturers and commercial breeders with a rapid method to screen their flocks for avian leukosis virus antibodies.

Proposed Course of Project: To continue with the improvement and development of immunofluorescence techniques for the detection of adventitious agents in human viral vaccines of egg origin.

Honors and Awards: None.

Publications:

Aulisio, C. G., and Shelokov, A.: Substitution of egg yolk for serum in indirect fluorescence assay for Rous sarcoma virus antibody. Proc. Soc. Exp. Biol. and Med. 126: 312-315, 1967.

Serial No. 124

1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on smallpox vaccines and vaccinia virus

Previous Serial Number: Same

Principal Investigator: Ruth C. Dunlap, Ph. D.

Other Investigators: Alexis Shelokov, M.D.
Judith K. Patt, M.S.

Cooperating Units: None

Man Years:
Total: 2.8
Professional: 1.8
Other: 1.0

Project Description:

Objectives: 1) To correlate vaccinia virus particle count of smallpox vaccines with other assay methods, viz., rabbit scarification, CAM pock titration and TC plaque assay; 2) To investigate certain aspects of virus biology, using vaccinia virus as a model; 3) To establish and put into operation an adequate electron microscope facility for work with vaccinia and other viruses.

Methods Employed: In addition to the usual techniques of viral propagation and assay, electron microscopy is being used routinely.

Major Findings: A 3-pronged attack on the problem of vaccinia virus particle content in smallpox vaccines has been used. We experimented with several enzymes to try to break up the extraneous debris contained in the vaccines. Incubation with 0.2% trypsin-chymotrypsin at 37 ° C for 1 hour proved the most successful method, but still left too much debris for accurate vaccinia virus counts. We then made successive extractions of virus from the vaccines using genetron. The amount of extracted virus was readily countable and from this, mathematical extrapolations could be made to estimate the amount of virus in the vaccine. A third approach has been to compare the biologic titer of the vaccine with that of virus extracted from the vaccine. The particle count of the extracted virus is readily ascertainable and a simple ratio provides the amount of virus in the vaccine.

Reactivation of heated poxviruses has been accomplished, using UV inactivated virus as the reactivating virus. UV treatment reduces the reactivating ability but this reduction is significantly less than the loss of infectivity caused by irradiation. Incidental to these studies was the discovery by electron microscopic observation of the heated virus that 60°C heat for 15 minutes disintegrates approximately 95% of the virus.

Studies have been carried out concerning the effect of aggregation of virus on biologic titer. With all the vaccinia virus strains we used, we found that aggregation increased the plaque titer, indicating that complementation of function or perhaps recombinational events possible with multiple infection of the cell, improved the ability of the virus to replicate.

A satisfactory working relationship with Melpar, Inc. has been established providing us with the use of a JEM electron microscope (see contract-narrative-Melpar, Inc.). We use this microscope for routine particle counts on all virus preparations used in our laboratory, in studying the effects of aggregation and dispersion and to give us visual observation of such factors as the viral content of smallpox vaccines, the degree of purity of virus suspensions, and the effect of various substances or treatments on virus structure. This facility has also been utilized by other members of LVR for visual examination of their specimens.

Significance to Bio-Medical Research and the Program of DBS: Determination of the viral content of smallpox vaccines provides a correlating factor to various biologic titers and would prove useful in the standardization of potency requirements of DBS. It is hoped that interest in this aspect of quantitation will encourage manufacturers to produce a cleaner product more amenable to this type of quantitation. Electron microscope examination provides a valuable extra parameter in all studies dealing with viral behavior.

Proposed Course of Project: This is a continuing project. Studies of biological interrelationship of the poxviruses will be extended, and electron microscopy methods applied to other viruses.

Honors and Awards: None.

Publications: None.

Serial No. 59
1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Antigenic studies of rickettsiae

Previous Serial Number: Same

Principal Investigator: Richard A. Mason, D.V.M., Ph. D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1.0
Professional: 0.3
Other: 0.7

Objective: The objective is to determine minimal antigenic and immunogenic quantities of Rickettsia rickettsii antigen in guinea pigs.

Methods Used: The immunity of guinea pigs vaccinated with increasing doses of a crude cell suspension containing R. rickettsii antigen was challenged with a standard dose of viable R. rickettsii. Rickettsial antigens used for vaccination were obtained from infected cell cultures. Specific antibodies to R. rickettsii were detected by means of a complement-fixation test.

Major Findings: Only a limited number of trials have been completed and the findings are preliminary and general in nature.

All guinea pigs that received the highest doses of vaccine developed specific antibodies and all such animals were resistant to infection with viable rickettsia. The lowest doses of vaccine failed to evoke detectable levels of antibodies and guinea pigs in these groups were as susceptible to infection as non-vaccinated guinea pigs. With intermediate doses of vaccine, some guinea pigs were resistant to infection with viable rickettsia while others of the same group showed signs of acute infection. Those animals that could be infected developed specific antibodies to R. rickettsii antigens after challenge while those that were resistant had no detectable antibodies either after vaccination or after challenge.

Significance to Bio-Medical Research and the Program of DBS: These studies will help determine whether CF antibody titers are reliable indicators of immunity to R. rickettsii infection and will establish the degree of correlation between the CF procedure and immunity in guinea pigs.

Proposed Course of Project: This project will be continued during the coming year.

Honors and Awards: None.

Publications: None.

Serial No. 82
1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of the nucleic acids and genetic relatedness of rickettsiae

Previous Serial Number: Same

Principal Investigator: Richard A. Mason, D.V.M., Ph. D.

Other Investigators: Emilio Weiss, Ph. D.
David T. Kingsbury, M.S.

Cooperating Units: Naval Medical Research Institute, NNMC, Bethesda, Md.

Man Years:

Total: 1.7
Professional: 0.3
Other: 1.4

Objective: The project is designed to characterize rickettsiae with respect to their DNA base ratios and to compare the genetic relatedness of rickettsiae and other intracellular parasites.

Methods Employed: Rickettsiae of several genera were grown in BS-C-1 cell culture, purified and used as sources of P^{32}-labeled or non-labeled DNA. For work with P^{32}-labeled organisms, enzymes were used in numerous attempts to minimize the number and length of manipulations associated with rickettsial purification. Rickettsial growth in cell cultures incubated with low-phosphate or "phosphate-free" media were studied. Low-phosphate medium consisted of phosphate-free culture media supplemented with regular calf serum. "Phosphate-free" medium was prepared similarly except that dialyzed calf serum was used.

Major Findings: Large quantities of DNA from two rickettsial species were isolated during the first half of the year. These were stored with previously accumulated DNA samples for use in homology trials with P^{32}-labeled DNA samples.

Rickettsiae grew satisfactorily in cultures with low-phosphate medium but uptakes of P^{32} from the medium into DNA were not adequate for homology trials. Studies using "phosphate-free" medium for P^{32} incorporation are not completed.

A simplified purification procedure was found but to date has only been used with small lots of infected culture. Only a small percentage of rickettsia remained with cell debris and the yield of intact rickettsia was satisfactory. The technique involved omission of celite treatment and treatment with DNAase and pronase.

Significance to Bio-Medical Research and the Program of DBS: Studies of rickettsial genetics and DNA are of importance as an additional means of understanding relationships among rickettsiae and in discovering features-in-common between these microorganisms and other intracellular parasites.

Proposed Course of Project: It is expected that homology trials will be done by the end of this fiscal year, or very early in the next fiscal year. These will be final experiments and will terminate this project.

Honors and Awards: None.

Publications: None.

Serial No. 65

1. Virology & Rickettsiology
2. Basic Virology & Rickettsiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanisms of intracellular infection

Previous Serial Number: Same

Principal Investigator: Richard A. Mason, D.V.M., Ph. D.

Other Investigators: None

Man Years:

Total: 1.3
Professional: 0.4
Other: 0.9

Project Description:

Objective: This is part of a continuing project designed to study biological and biochemical factors associated with intracellular parasitism. The present study was continued from last year as planned. The purpose of this phase of work is to define the nutritional requirements of the intracellular parasite Rickettsia quintana. Progress made will be useful in studies of more fastidious intracellular parasites.

Methods Employed: The ability of systematically simplified media to support growth of R. quintana was studied. Major efforts were directed toward defining essential growth factors in yeast extract and crude red blood cell lysate (Difco hemoglobin). In addition, the growth cycle of R. quintana in liquid medium was determined by titrations of numbers of organisms on solid medium.

Major Findings: Data were obtained which suggested that hemoglobin may not be the sole nutritional requirement provided by lysed red blood cells. With one agar-medium, growth of the organism occurred when red blood cell lysate (Difco hemoglobin) was included but did not occur when twice-cytstallized hemoglobin was used. Hemoglobin alone (twice crystallized) was also unable to substitute completely for red blood cell lysate when used in a second agar-medium. In this instance numbers of colonies were reduced by 30% when hemoglobin was employed.

Yeast extract was required for growth of R. quintana in all media tested. Amino acids, vitamins and salts used alone or in various combinations were unable to substitute for yeast extract. Fetal calf serum was not able to completely replace yeast extract.

A liquid medium containing fetal calf serum (FCS) but not calf serum (CS) was previously reported capable of supporting growth of R. quintana. The medium contains three simple constituents other than FCS and these can be combined and autoclaved. The growth supporting factor(s) in FCS was: stable for at least several months at -20°C, stable for several days at 4°C, and stable at 56°C for 30 minutes. It was partially inactivated at 100°C in 2 minutes and completely inactive after 10 minutes at 100°C. The essential factor(s) is not dialyzable. Stationary cultures were incubated at 35-36°C under 5% CO_2 at 90% relative humidity. Under these conditions titers increased from initial levels of approximately 10^3 colony-forming units (CFU)/ml to more than 10^8 CFU/ml in 5 days. The lag phase was approximately 24 hours and was followed by 72 hours of exponential growth. The generation time in liquid culture was approximately 4 hours.

Significance to Bio-Medical Research and the Program of DBS: Basic studies of host-parasite interaction are a necessary parallel to the development of systems useful for vaccine production and related testing procedures.

Proposed Course of Project: This project will be continued at a reduced level in the coming year.

Honors and Awards: None.

Publications: None.

Serial No.67

1. Virology & Rickettsiology
2. Tissue Culture Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Standardization techniques applicable to large-scale production of cell cultures

Previous Serial Number: Same

Principal Investigator: Hector Montes de Oca, M.D. (7/67-12/67)
Henry C. Orr, Ph. D. (1/68-date)

Other Investigators: Alexis Shelokov, M.D.
Joseph L. Rogers

Cooperating Units: None

Man Years:

Total: 0.5
Professional: 0.3
Other: 0.2

Project Description:

Objectives: To implement new and improved techniques and methods developed in the Section and/or elsewhere in large-scale production of cell cultures.

Methods Employed: Information regarding various techniques and methods as related to large-scale production of cells is gathered from the literature and by consultation. This information is evaluated and applied in pilot studies to production of large quantities of cells. Upon successful application, these improved and tested techniques are standardized as part of the Tissue Culture Section procedures for producing quantity-cell cultures of high quality for research and vaccine testing purposes.

Major Findings:(1) Use of laminar-flow work benches does improve asceptic working conditions. (2) Utilization of powdered media provides greater standardization of such reagents than heretofore possible with liquid media prepared when needed. (3) Use of the Counter counter provides greater resproducibility of cell counts. (4) Brockway 32-oz bottles are superior to Modern Oval bottles in uniformity and other aspects for maintenance of stock cultures. 5. Selas-filtered sera are superior to Millipore-filtered sera since the absence of detergents in Selas-filtered material enhances cloning of certain cells. (6) The Kahlenberg semi- automatic

dispersing machine can be employed in seeding cultures in tubes saving many man-hours of work. (7) The issuance of a work manual of our operating procedures ensures better standardization of cell culture preparation and maintenance. (8) The liquid medium for PPLO-sterility checks gives much more rapid results than solid plates and allows faster evaluation of cells to be furnished to DBS investigators.

Significance to Bio-Medical Research and the Program of DBS: In order to assure reproducibility of results obtained by investigators in their control and research studies, standardization of our cell culture techniques and systems is of paramount importance.

Proposed Course of the Project: This effort will be continued during the coming year.

Honors and Awards: None.

Publications: None.

Serial No. 79

1. Virology & Rickettsiology
2. Tissue Culture Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: **Development of new cell culture systems as possible substrates for vaccine production and/or testing**

Previous Serial Number: Same

Principal Investigator: Henry C. Orr, Ph. D.

Other Investigators: Joseph L. Rogers
George P. Probst
James Baker
Naomi T. Stocks
Nicola M. Tauraso, M.D.
Samuel Baron, M.D.

Cooperating Units: Laboratory of Pathology, DBS
Immunoserology Section, LVR
Laboratory of Biology of Viruses, NIAID

Man Years:

Total: 0.5
Professional: 0.1
Other: 0.4

Project Description:

Objectives: To develop new established cell lines as additional substrates for vaccine production and/or testing.

Methods Employed: By using various cell culture techniques as may be appropriate, the isolation, passage, characterization and preservation of cell cultures from such seldom used tissues as brain, thymus, bone marrow, etc. of nonhuman origin are made.

Major Findings: Much of this work is still very preliminary. However, primary, secondary and tertiary cell cultures have been prepared from the following tissues of rabbits and embryonic calves: thymus, spleen, liver, heart and lung. In addition, spinal cord from African green and liver cells from rhesus monkeys have been initiated in culture. The method of dispersion of cells from the tissues appears to determine the morphologic type of cell which grows.

In a collaborative study, it was tentatively found that primary cultures of various rabbit tissues were differentially sensitive to the action of interferon. Aliquots of these same tissues supported growth of representative viruses from various groups to varying degrees.

Significance to Bio-Medical Research and the Program of DBS: Additional well defined *in vitro* host systems are needed for vaccine production and for testing. Especially desirable at this time are cell lines in which certain fastidious agents such as Burkitt's virus, scrapie agent, and others can be passed, studied and bio-assayed.

Proposed Course of Project: To continue attempts to establish cells in culture from a number of organs from various animals including cold-blooded vertebrates. Collaboratively to examine these cells for adventitious agents, for their susceptibility to selected members of various virus groups, to evaluate the sensitivity to and production of interferon, as well as cell ploidy.

Honors and Awards: None.

Publications: None.

Serial No. 83
1. Virology & Rickettsiology
2. Tissue Culture Section
3. Bethesda, Md.

PHs-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the biology of cultured cells

Previous Serial Number: Same

Principal Investigator: Hector Montes de Oca, M.D. (7/67-12/67)
Henry C. Orr, Ph. D. (1/68-date)

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 0.4
Professional: 0.3
Other: 0.1

Project Description:

Objectives: As a result of cross-fertilization between the research and production units in the Section, to carry out short-term studies on the biology of the cultured cells. Currently, we are examining or have examined the following:

1. Modulation of cell culture populations induced by trypsin, hypotonic medium and other cultural conditions.

2. The trypsin-versene method of cell dispersion.

3. Mechanism of action of DMSO as a preservative for freezing cells.

4. Beneficial effects of substituting galactose for glucose in cell culture medium.

Methods Employed: Tools of the cell biologist such as cytochemistry, time-lapse cinematography, phase contrast and fluorescent microscopy are used in investigating these problems.

Major Findings: 1. During attempts to evaluate the MS&D smallpox vaccine using the BS-C-1 cell, it became apparent that cells used by MS&D were morphologically different from those maintained at DBS. It was found that the company routinely subjected their cells to a longer period of trypsinization than that customarily used for removing cells from glass.

When this method of trypsinization was used on BS-C-1 cells from DBS, they shifted morphologically to resemble the MS&D cells. In addition, shorter periods of trypsinization of the MS&D cells caused them to revert to the appearance of our BS-C-1 cells. This observation has caused us to examine more closely the modulative role that trypsin might play on cell populations.

We have cultured cells from rabbit lung by two methods. A portion of lung tissue was trypsinized and seeded into 32 oz bottles. The remainder of this same tissue was finely minced and placed into other bottles. In the case of the trypsinized lung, a culture resulted which consisted exclusively of fibroblasts; epithelial cells grew from the minced tissue. These cell populations have been maintained for some 5 or more generations-the--the fibroblasts are passed by the trypsinization method of cell dispersion and the epithelial cell cultures by scraping. Viral susceptibility, cytochemical properties and cloning efficiency which may accompany morphologic modulation are being examined.

2. It has been found that the combination of trypsin and versene for dispersion of cells from tissie results in a much greater cell yield than that obtained by trypsin alone. As reported last year, many commercial tha houses have now adopted this method for dispersing cells from tissue with excellent results. This finding, reported as a preliminary note, will be soon submitted as a full paper for publication.

3. It appears that a cytotoxic factor can be removed from DMSO by prior absorption with viable cells. (Removal of this factor enables nematods to be recovered from the frozen state in DMSO).

4. The life-span of the WI-38 cells has been shown to be increased by substituting galactose for glucose in the culture medium.

Significance to Bio-Medical Research and the Program of DBS: Information gained from studies in this project provides the direct and immediate basis on which changes in methodology are made in keeping with the goals of the Section in producing high quality cell cultures. These results are directly applicable to cell culture production.

Proposed Course of Project: This is a continuing project and current studies are developed and investigated in the light of greater knowledge, need and feasibility.

Honors and Awards: None.

Publications: None

Serial No. 142
1. Virology & Rickettsiology
2. Tissue Culture Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Identification of specific in vitro cell differential antigens

Previous Serial Number: None

Principle Investigator: Henry C. Orr, Ph. D.

Other Investigators: Alexis Shelokov, M.D.

Cooperating Units: None

Man Years:

Total: 0.3
Professional: 0.2
Other: 0.1

Project Description:

Objectives: To demonstrate in cell cultures, a specific antigen(s) which can serve as a marker for cell identification and characterization.

Methods Employed: Methods and techniques are employed to find a "clean" specific immunizing cell antigen. Such antigen to be used to obtain specific antiserum. These methods include isolation of various cell fractions by biochemical approaches, characterization of these fractions by physical and immunochemical techniques, preparation of antisera and subsequent localization and detection of cell antigens by immunofluorescence.

Major Findings: This is a new project just getting underway. The WI-38, Chang's liver, MA-104, and BS-C-1 cells have been fractionatedi into the following components: 1) a lysate residue, 2) a lysate fraction, 3) a cell "globulin" fraction precipitable by $(NH_4)_2 SO_4$ and 4) a $(NH_4)_2 SO_4$ nonprecipitable residue. These have been dialyzed, centrifuged at high speed, protein concentration determined and will be inoculated into rabbits for production of antiserum, shortly.

Significance to Bio-Medical Research and the Program of DBS: Cell culture typing by specific differential-markers is important from the

point of view of detecting cross-cellular contamination and possible host cellular contaminants of vaccines produced in tissue culture.

Proposed Course of Project: Cell surface antigens, internal cellular antigens and cell products as antigens will be examined by immunocytochemical techniques in evaluating antigenic procedures for cell culture identification and characterization.

Honors and Awards: None

Publications: None

Serial No.60
1. Virology & Rickettsiology
2. Section on Respiratory Viruses
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies concerned with influenza viruses

Previous Serial Number: Same

Principal Investigator: J. A. Morris, Ph. D.

Other Investigators:
Michael F. Barile, Ph. D.
Henry H. Bloom, Ph. D.
Paul Brown, M.D.
Ottis Causey, M.D.
Jose David-Ferreira, M.D., Ph. D.
Carleton Gajdusek, M.D.
Clarence J. Gibbs, Ph. D.
Elizabeth Jackson, B.A.
Paul B. Johnston, Ph. D.
Edwin H. Lennette, M.D.
Maurice A. Mufson, M.D.
John Parker, Ph. D.
Aime Pellissier, M.D.
Wallace P. Rowe, M.D.
Charles L. Wisseman, M.D.

Cooperating Units:
Laboratory of Bacterial Products, DBS
Microbiologics Associates, Inc.
Laboratory of Child Growth & Development, NINDB
The Rockefeller Foundation (Ibadan)
Fundacao Gulbenkian (Oeiras)
Laboratory for Study of Slow, Latent & Temperate Viruses, NINDB
Harvard University
University of Louisville
California State Department of Public Health
University of Illinois
Institut de Pasteur (Brazzaville & Nice)
Laboratory of Viral Diseases, NIAID
University of Maryland

Man Years:

Total: 1½
Professional: 3/4
Other: 3/4

Objectives: Work on this project was directed toward collection of information from many biologic study systems which will be of assistance in improving effectiveness of respiratory virus vaccines.

Q-fever rickettsiae as an immunologic adjuvant. One of the purposes of this study was to explore the feasibility of combining killed Q-fever rickettsiae (Coxiella burneti) with inactivated epidemic typhus vaccine to produce a single product which would yield an acceptable antibody response to both components. This not only was accomplished for both components when guinea pigs and man were vaccinated with the combined R. prowazeki--C. burneti preparation but also yielded striking evidence that the Q-fever component served as an effective adjuvant in guinea pigs and in man for the epidemic typhus antigens. This study, with its purpose accomplished, was completed and a detailed interpretative report was published.

Immunologic preparative effect of small doses of vaccine and studies on influenza in immunologic virgin populations. Six years ago 3 studies were undertaken to gather information to add to the surprisingly little that is recorded in the literature concerned with the immune response of man and animals to multiple exposures of varying amounts of a given antigen in the live or dead state. The studies were designed to attack this broad biologic problem from 2 vantage points: rickettsial (epidemic typhus) and viral (influenza). Studies concerned with the immune response to rickettsial antigens are complete and the findings are described in detail in 2 interpretive publications.

Previously reported work concerned with the influenza aspects of the study yielded information which appeared to support the contention that influenza vaccines achieve greatest effectiveness in reducing clinical disease when they contain optimum quantities of antigen derived from contemporary strains of influenza virus. During 1966 a study was initiated among the inhabitants of Micronesia to test the efficacy of single and multiple doses of inactivated monovalent and polyvalent influenza vaccines containing "old" and contemporary influenza type A and type B virus strains. An influenza epidemic caused by type B virus occurred in the study population 3 months following vaccination. The serum neutralizing antibody response to the A2 and B influenza virus vaccines was of the expected order but the excellent serologic response to the vaccines was not associated in the study group with a reduction in the incidence of clinical disease when this was compared with appropriate control populations. This observation was both surprising and unexpected. Accordingly, studies were begun 2 years ago to gather information to provide a definitive explanation for the observed vaccine failure and these have continued throughout the year. For completion these studies will require an additional $\frac{1}{2}$ year of effort to accomplish the required serologic testing and to prepare for publication a report describing and interpreting the findings.

Viruses resistant to the action of chemical and physical agents: The principal aim of this study was to collect information which might add to an understanding of the brain pathology induced by the scrapie agent in the experimentally infected mouse. The study was undertaken because the scrapie agent represents a family of infectious agents which are present in the tissues of animals (and probably man) which are not inactivated by prolonged exposure to formalin and ultraviolet light. In the present work certain structures were encountered in scrapie infected mouse cortex that were not seen in an extensive examination of appropriate control materials. These structures were described in detail and an attempt was made to interpret the significance of their presence in infected mouse brain in a published report. This project has now been enlarged to incorporate into the study scrapie sheep brain and kuru chimpanzee and human brain.

Studies on viruses recovered from and capable of producing in man illnesses indistinguishable clinically from influenza. Dr. Ottis Causey (The Rockefeller Foundation Laboratories, University of Ibadan) in September, 1964, recovered a transmissible agent in mice inoculated with plasma obtained from a 6-month-old child with a febrile, grippe-like illness. In the following 2 months he recovered in mice inoculated with 850 clinical specimens and from the tissues of uninoculated mice 65 strains of the same agent. In the course of this work 2 laboratory workers contracted influenza-like-illnesses and from the bloods of both workers strains of the same agent were recovered. It was concluded by the workers in the Rockefeller Laboratories at Ibadan that the unidentified agent was present as a latent infection in the mouse colony. In early 1965, 10,000 mice were immunized against the infection by the intraperitoneal injection of an experimental vaccine prepared from infected mouse carcasses. In early 1967 assistance was requested in the identification of the recovered agents. The agents were readily identified in tests employing reagents provided by Dr. Causey as strains of Coxsackie A virus. Further study of the reagents yielded results that suggested that the virus was introduced into the laboratory in the blood of the 6-month-old child and thereafter it spread to the mouse colony where it became established as an enzootic infection. These events provided evidence to demonstrate again that Coxsackie A virus may cause grippe-like disease in man and that man may contract the infection by exposure to infected mice. While observations that first led to these conclusions were made more than 10 years ago, the recent findings are of significance because they re-emphasize the importance of awareness of Coxsackie A virus as a cause of laboratory infections.

As reported earlier, Pellissier and Lumaret in 1948 recovered in guinea pigs a transmissible agent from the blood of a patient with African epidemic icterus. Subsequently strains of this agent were recovered in Africa from patients with an anicteric, influenza-like, type of the disease. As previously reported the recovered agents were shown to be members of the hepatoencephalitis virus group. Because of its unique behavior in guinea pigs and in mice and in serologic tests with hepatoencephalitis virus reagents, efforts have continued to characterize further the African epidemic ictera virus. On the basis of serologic data it appears that the African epidemic icterus

agent contains antigens that are also present in lymphocytic choriomeningitis virus. Studies to elucidate this surprising finding are in progress.

Characterization of viruses and other microbial agents encountered in test animals and in cell cultures employed in testing of biologics. It has been suggested that an infectious agent, possibly the rabbit syncytium virus, is present as a contaminant in the simian-foamy-virus type-4 reagents which were prepared in cultures of rabbit kidney cells on contract for the National Cancer Institute. In collaborative work with Dr. Paul B. Johnston studies were initiated to identify the contaminating virus.

A previously undescribed, serologically distinct, species of mycoplasma was recovered from the brains of sheep and mice and subsequently from cell cultures of human, chimpanzee and canine origin. The role that the new species of mycoplasma plays in the ecology of its animal hosts is the subject of current work, but by its presence as a contaminant in cell cultures of diverse origin, this organism assumes importance in that it constitutes yet another troublesome cell culture contaminant to be recognized. A full description of this organism is found in the report by Dr. Michael F. Barile, Laboratory of Bacterial Products, and in a manuscript prepared for publication.

Proposed Course of the Project: Change in the antigenic composition of influenza viruses constitutes a problem of first importance in maintenance of adequate control of influenza by vaccination. This requires continuation of the established world-wide surveillance of influenza virus to detect soon after their appearance new influenza virus variants. This work will continue as a cooperative effort of collaborative workers throughout the world.

Laboratory differentiation of influenza and influenza-like-illnesses is an essential continuing study. Continuation of this study will make it possible to accumulate information that will be of assistance in eliminating or, at least, in reducing in importance a complicating factor in interpretation of influenza virus vaccine protection data and of other epidemiological information related to influenza in man.

The definitive characterization of transmissible agents encountered in the tissues of animals used in the production or testing of vaccines will continue to be of assistance in overcoming manufacturing problems and in defining variables which are inherent in vaccine potency assay systems.

Honors and Awards:

1. Participant (Dr. Morris)--Seminar on Slow Viruses, Oeiras, Portugal, July 1967.

2. Invited speaker (Dr. Morris)--Panel discussion on slow viruses, National Institute of Neurological Diseases and Blindness, New Iberia, Louisiana, March, 1968.

3. Invited speaker (Dr. Morris)--Current interests in slow viruses, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, April, 1968.

4. Program advisor and consultant (Dr. Morris)--Slow, latent, and temperate virus infections, National Institute of Neurological Diseases and Blindness, NIH.

5. Program advisor and consultant (Dr. Morris)--Respiratory virus study group, World Health Organization.

6. Program advisor and consultant (Dr. Morris)--Respiratory virus study group, Turkish Army, Medical Command (Dr. M. Saglam, Director).

7. Program advisor and consultant (Dr. Morris)--Laboratory of cell biology, Gulbenkian Foundation, Oeiras, Portugal.

8. Consultant (Dr. Morris) to the Scientific director for etiology, National Cancer Institute.

9. Consultant (Dr. Morris) Laboratory of Biologic studies, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

10. Member, Commission on Influenza, Armed Forces Epidemiological Board.

11. Member, Intercommission Committee on Standardization of Adenovirus Vaccines, Armed Forces Epidemiological Board.

Publications:

Morris, J. A., Wisseman, C. L., Aulisio, C. G., Jackson, E. B., Smadel, J. E.: Combined epidemic typhus and Q-fever vaccine: adjuvant effect of Coxiella burneti. Proc. Soc. Exp. Biol. and Med. 125: 1216-1220, 1967.

Morris, J. A., Jackson, E. B., Shaw, C. W. and Smadel, J. E.: Immunologic preparative effect in guinea pigs of small doses of Rickettsia prowazeki, strain E. Am. J. Epidem. 86: 746-755, 1967.

David-Ferreira, J. F., David-Ferreira, K. L., Gibbs, C. J., Morris, J. A.: Scrapie in mice: ultrastructural observations in the cerebral cortex. Proc. Soc. Exp. Biol. and Med. 127: 313-320, 1968.

Serial No. 98
1. Virology & Rickettsiology
2. Respiratory Virus
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies concerned with adenoviruses

Previous Serial Number: Same

Principal Investigator: J. A. Morris, Ph. D.

Other Investigators: Joseph A. Bell, M.D.
Walter Dowdle, Ph. D.
D. Carleton Gajdusek, M.D.
George G. Jackson, M.D.
Robert Peckinpaugh, M.D.
Mehmet Saglam, M.D.

Cooperating Units: National Communicable Disease Center
Laboratory of Child Growth and Development, NIMDB
University of Illinois
NAMRU-4
Medical Command, Turkish Army (Ankara)

Man Years:
Total: ½
Professional: ¼
Other: ¼

Project Description:

Objectives: The objective of this project is the accumulation of information which will be of help in re-establishing adenovirus vaccines to the armamentarium of respirovirus vaccines for use in man, and in addition, will be of assistance in understanding the biology of adenovirus infection.

Serologic and clinical responses in man and animals to viral components in adenovirus vaccines. The aspect of this project which was concerned with the serologic responses in man to the adenovirus and SV40 components in adenovirus vaccines was successfully concluded and the findings were published. The aspects of the project concerned with the serologic and clinical responses in hamsters and monkeys to the SV20 and to the SV20-SV-40-complex are now in progress.

An improved inactivated monovalent adenovirus type 4 vaccine. It was previously reported that studies were successfully carried out which resulted in the development of an inactivated adenovirus type 4 vaccine which was derived from virus propagated in human cell cultures. The new vaccine was shown to be free of SV40 antigen and its use in a recruit population provided a high degree (92%) of protection against adenovirus type 4 disease, but the type 4 virus was almost immediately replaced in the vaccinated population by adenovirus type 7. Therefore, the overall objective of this work to reduce to incidence of adenovirus disease in the test population during the study period was not accomplished. It appears advisable, on the basis of the available information, to delay the use of the adenovirus type 4 vaccine in recruit populations until such time as there is available a satisfactory adenovirus type 7 vaccine and studies to accomplish this are in progress.

Recovery of previously undescribed adenovirus types from Turkish army recruits with acute respiratory illness. Two new types of adenovirus were recovered from throat washings obtained from patients with primary atypical pneumonia in an epidemic of acute respiratory illness which occurred in Turkish army recruits in early 1967. The new viruses share common complement fixing antigens with known adenovirus types but the new viruses are not neutralized by antisera to any of the known adenoviruses of avian, murine or primate origin. The new types are currently under intensive study, to define their importance in human diseases and to determine their oncogenic potential in an experimental hamster system.

Failure to establish etiologic relationship between adenovirus and human hepatitis or to induce illness in chimpanzees by inoculation of human hepatitis material. It has been reported that adenovirus type 16 was recovered from the bloods of patients hospitalized with infectious hepatitis with a frequency that exceeded that which would be expected by chance (Science 152: 1390, 1966). In an attempt to determine the significance of this report studies were undertaken several years ago in collaboration with Dr. Joseph A. Bell to examine serial sera obtained before and after the onset of hepatitis and other diseases for presence of antibody to adenovirus type 16. On the basis of data which are now complete it appears that adenovirus type 16 was not etiologically related to hepatitis that occurred in our study group. There was no significant difference in either the frequency of occurrence or level of adenovirus type 16 antibody in the serum of the hepatitis and non-hepatitis patients. Further, no rises in serum level of adenovirus type 16 antibody were detected in the hepatitis patients during the course of their illnesses.

The frequency with which hepatitis occurs among chimpanzee handlers has resulted in renewed interest in the chimpanzee as an experimental host for this disease in spite of the fact that failures have resulted in all reported attempts to transmit human hepatitis to this age. In our attempt, known icterogenic human plasma was inoculated by multiple routes into 11 weanling chimpanzees and the inoculated animals were observed for 1 year

during which time a battery of liver function tests were performed at 14-day intervals. The results may be briefly summarized: all 11 chimpanzees remained healthy and the results of liver function tests remained within the normal range.

Proposed Course of the Project. Adenovirus vaccines manufactured in the period 1956-1963 were prepared in cultures of rhesus monkey kidney cells that were contaminated with a number of viruses including SV20, a simian adenovirus. This monkey virus is highly oncogenic in suckling hamsters. Moreover, antibody directed against SV20 is frequently found in man. Other than this, little is known of the biology of this virus. Accordingly, studies are underway to learn of the biologic behavior of live SV20 virus in monkeys and of the antigenic behavior of formalin-killed SV20 in man.

Honors and Awards:

1. Invited participant (Dr. Morris)--Symposium on Etiology of Infectious Hepatitis, Chicago, November, 1967.

Publications:

Morris, J. A., Shaw, C. W., Jackson, G. G.: Serologic response in man to adenovirus and SV40 components in adenovirus vaccines. Proc. Soc. Exp. Biol. and Med., 125: 751-756, 1967.

Serial No. 74
1. Virology & Rickettsiology
2. Section on Virus Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on antiviral and antitumor agents

Previous Serial No: Same

Principal Investigator: Chen Pien Li, M.D.

Other Investigators: Benjamin Prescott, Ph. D., NIAID
Bernice E. Eddy, Ph. D.
Michael L. King, Ph. D.
Elmer C. Martino

Cooperating Units: Laboratory of Infectious Diseases, NIAID
National Medical Defense Center, Taipai, Taiwan

Man Years:
Total: 4.0
Professional: 1.0
Other: 3.0

Project Description:

Objectives: It was proposed in the Annual Report 1967 that the study of antitumor activity be continued in the following aspects: (1) Further purification of the clam liver extract; (2) further study of prophylaxis of adenovirus-12 oncogenesis in hamsters: (3) pharmacological studies of the clam extracts; and (4) prevention of viral transformation of normal cells into malignant cells *in vitro* by clam liver extract.

Methods Employed: These have been described previously.

Major Findings: (1) The clam liver extract (lot D1058) prepared previously has been further purified by ultracentrifugation at 30,000 rpm for 2 hours. The antitumor activity was in the sediment and was increased 2-4 fold. Chemical studies indicated that the active principle might belong to the class of glycoproteins; (2) Clam liver extract was prepared in a new way. Fresh clam livers were treated with versene solution and then centrifuged. The sediment which contained the cells from the liver contained the antitumor activity. A fraction prepared from the clam liver containing mostly nuclear material did not show antitumor activity; (3) Clam liver extract (lot 1058) inhibited tumor formation in hamsters induced by adenovirus 12 more efficiently than the extract made from the whole clam.

It was therefore confirmed that adenovirus 12 oncogenesis in hamsters can be prevented; (4) The clam extract (lot 1058) when mixed with herpes simplex virus at 37 C for 24 hours reduced the viral titer by 2-3 logs. It is concluded that the clam liver extract can inactivate herpes virus in vitro but only very slowly; (5) The activity of the clam liver extract was different from that of certain bacterial polysaccharides reported by M. J. Shear et al. Their material induced hemorrhagic lesions in sarcoma 37 in mice and possessed no direct cytotoxic effect on the tumor cells in vitro. The clam liver extract did not produce hemorrhage in sarcoma 180 in mice in our experience but showed strong cytotoxic effect on tumor cells in culture. However, contamination with other type of bacterial products including L-asparagenase cannot be ruled out.

Significance to Bio-Medical Research and the Program of DBS: The clam liver extract possessing antitumor activity can be used at present as an experimental tool. Further study might lead to some other practical implication. The present study also established that adenovirus 12 oncogenesis in hamsters can be prevented. This was probably accomplished by interference with the process of transformation of normal cells into malignant cells.

Proposed Course of Project: The possible presence of L-asparaginase in the clam extract and the possibility of prevention of the transformation of normal cells into malignant cells by adenovirus 12 and/or other viruses will be studied.

Honors and Awards: None

Publications: None.

ADJUNCTS

To The Office Of The Director

Serial No. DBS 01
1. Experimental Virology
2. Office of the Director
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Experimental Virology

Previous Serial Number: Same

Principal Investigator: Bernice E. Eddy, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	3.0
Professional:	1.0
Other:	2.0

Project Description:

Objectives: To investigate the safety of biological products, especially in relation to the oncogenicity of viruses and means of preventing neoplastic development; investigate new viruses and other agents in relation to their importance in human disease and for the purpose of exploring the possibility of preventing diseases that at present are uncontrolled.

Methods Employed: Conventional methods of propagating viruses in cell cultures and in animals, serological and cellular determination of antigenic relationships and pathological examination of tissues.

Major Findings: A continuing investigation in this section relates to the question of viral oncogenesis and to the circumstances under which neoplastic changes in cells can be enhanced or suppressed.

Newborn offspring of hamster females, but not hamster males, which had been infected with adenovirus type 12, were resistant to tumors when inoculated with adenovirus type 12.

Certain plant extracts or endotoxin from E. typhosis, when given along with adenovirus type 12 or with SV40 to hamsters when had been infected when newborn with homologous virus, had only a slightly enhancing effect on immunity as compared to control animals.

A line of continuous human lymphoma cells, which contain virus particles when examined in the electron microscope, on injection into hamsters produced central nervous system symptoms and death in a few animals. A virus, as yet, has not been recovered from these cells, or from other human tumors which are being studied.

Significance to Biomedical Research and the Program of the Division: This program is of major significance in developing an understanding of hazards that may exist in proposed biological products and in providing information on the importance of the relationship of immunity and oncogenesis.

Proposed Course of Project: Work will be continued on immunologic effects in relation to oncogenicity of various viruses, employing techniques and experimental protocols similar to those above but varied in different parameters.

(With C. P. Li and others) Studies concerning anitumor effect of clam extracts on hamster tumors are being continued.

Honors and Awards:

Superior Service Award, U. S. Department of Health, Education and Welfare.
Life membership in Sigma Delta Epsilon.

Publications:

Eddy, B. E.: Viruses and cancer. Journal Formosan Medical Association 66:369-381, July 1967.

Eddy, B. E.: Viruses and cancer. Sigma Delta Epsilon News 31: 7, May 1967.

Eddy, B. E.: Virus diseases. In Williams, R. J., and Lansford, E. M., Jr. (Eds.): Encyclopedia of Biochemistry. New York, Reinhold Publishing Corporation, 1967, pp. 823-825.

Annual Report of the Biometrics Section
Division of Biologics Standards
July 1, 1967 through June 30, 1968

I MISSION

The primary responsibility of the Biometrics Section, Division of Biologics Standards, is to insure that efficient statistical techniques are available and used in the scientific operations of the Division. For the most part, adequate techniques are available in the literature, but on occasion this will not be so. Even where appropriate techniques have been discussed, the literature may be scattered, highly technical, and contradictory, requiring study and comparison for adequate exploitation.

II ANALYSES SUMMARY

In Fiscal Year 1968, 42 specific tasks were initiated in the Section and 10 were continued from the previous fiscal year. Of these, 28 were completed, 11 were cancelled or suspended, and 13 are continuing. (Table I).

III METHODOLOGICAL RESEARCH

1. A comprehensive review of the performance of the official potency test for tuberculin has been under way for some time. Results to date were reported at the Conference on Evaluation of Procedures for Tuberculin Testing in February of 1968. More recent data is under study. It is expected that the completed results will be published.

2. Limited data on the reaction of humans to the intradermal injections of histamine dilutions were studied to evaluate the comparative advantage of using (1) cross diameter products (CDPs) or cross diameter sums (CDSs) as response metameters, and of (2) measuring the diameters of the wheal or the erythema. A report embodying the results has been prepared by Harold Baer and Clifford J. Maloney.

3. Data were studied on the homologous and heterologous delayed sensitivity reaction of one representative from each of six species of tuberculins. After adjustment of challenge dose to obtain a response to the homologous organism as near as possible in the range from 150 to 200 cross diameter product (CDP), the 36 reciprocal reaction results yield a 6 X 6 matrix of readings in which the readings on the main diagonal are those from the homologous experiments. The appearance of this matrix suggested that, conceivably, the six species could be ranked in a linear order on the basis of their overlap in heterologous response. The fit appeared gratifyingly close. The conclusions were incorporated in a paper by S. D. Chaparas in the

Conference on Evaluation of Procedures for Tuberculin Testing in February 1968.

4. Results of nearly 18,000 simultaneous and Tine PPD tests submitted by a manufacturer were analyzed in connection with an application for a product license. Two interesting phenomena were observed. The first was that the incidence of false negative and false positive test results varied widely between investigators. The second was that, by either test, the population increase of tuberculin incidence rises on a fairly smooth growth curve from approximately 3% in the first to approximately 40% in the seventh decade of life. The novelty of either observation has yet to be verified.

IV OPERATIONAL PROBLEMS

Progress on two studies described in the Fiscal Year 1967 Annual Report was in abeyance awaiting program development in the Computer Research and Technology Division. Some new results on one, exact chi-squares for 3 X 3 contingency tables, appeared at the end of the report period.

The suitability of a remote station, particularly for the prompt computation of routine statistical analyses, was investigated and recommendation for the application of such a facility submitted.

V FUTURE PLANS

The four subtasks specified in Section III have been added to the carryover from the FY 1967 report. In most cases progress depends on Computer Division support and/or the acquisition of a within house capability.

TABLE I

SUMMARY OF STATISTICAL ANALYSES FOR FISCAL YEAR 1968

S.A.N.	DATE INITIATED	DATE COMPLETED	TITLE	RESEARCHER
105	Apr. 1966	Cancelled	POLYCHOTOMOUS ASSAY	Dr. Maloney
114	Oct. 1966	Nov. 1966	RABIES ACCEPTANCE SAMPLING	Dr. Seligmann
115	Aug. 1966	Preliminary report 2/9/67, Add'l report 10/18/67.	TEST OF FLU VACCINES	Dr. Wagner
118	Nov. 1966	Cancelled	INDEXING OF CANCER INSTITUTE MONOGRAPH 21	Dr. Maloney
125	Feb. 1967	Cancelled	RUBELLA ACTIVE FRACTION	Dr. Amstey
127	Feb. 1967		EVALUATION OF A STANDARD TUBERCULIN TEST, PART III	Mr. Kolb
129	Mar. 1967	Cancelled	RUBELLA ACTIVE FRACTION, PART II	Dr. Amstey
130	Mar. 1967	June 1967	Q-FEVER RICKETTSIAL & TYPHUS VACCINE	Dr. Morris
132	Mar. 1967	Cancelled	TWEEN 80 EFFECT	Dr. Hobbins
133	Apr. 1967	Suspended	GROWTH CURVES	Dr. Godfrey
134*	Apr. 1967	Apr. 1967	BURKITT TITERS	Dr. Gerber
135*	Apr. 1967	May 1967	ALLERGY STANDARDS	Dr. Godfrey
136*	May 1967	May 1967	PATHOGENESIS OF LESIONS--BCG	Dr. Chaparas
137*	May 1967	Aug. 1967	PLASMA STUDY	Dr. Rymer (Dr. Tripp)

TABLE I - Continued

SUMMARY OF STATISTICAL ANALYSES FOR FISCAL YEAR 1968

S.A.N.	DATE INITIATED	DATE COMPLETED	TITLE	RESEARCHER
138*	May 1967	May 1967	GEOMETRIC MEANS OF BURKITT SERA	Dr. Gerber
139*	May 1967	Mar. 1968	OT & PPD REACTIONS	Dr. Chaparas
140*	June 1967	Aug. 1967	SUB-AGENT COMPARISON	Dr. Godfrey
141*	June 1967	Suspended	RUBELLA PASSAGE VS. HAI ANTIBODY	Dr. Parkman
142*	June 1967	Ref. S.A.N. 150	GROWTH MODIFICATION	Dr. Montes de Oca
143*	June 1967	Cancelled	FORM OF DISTRIBUTION CURVE	Dr. Hobbins
144	July 1967		TUBERCULIN REGRESSION COMPARISON	Dr. Baer
145	July 1967	Oct. 1967	TUBERCULIN STABILITY, PART I	Dr. Baer
146	July 1967		BRANCHED CHAINS	Dr. Baer
147	Aug. 1967	Cancelled	NATURAL RUBELLA & VACCINATION ANTIBODY	Dr. Parkman
148	Aug. 1967	Jan. 1968	HISTAMINE RESPONSE	Dr. Baer
149	Aug. 1967	Suspended	VIRUS COUNT	Dr. Hobbins
150	Aug. 1967	Oct. 1967	GROWTH MODIFICATION, PART II	Dr. Montes de Oca
151	Aug. 1967	Oct. 1967	POLIO VACCINE STANDARDS	Mrs. Eva Brown
152	Sept. 1967	Mar. 1968	FOCI COUNT	Dr. Hobbins
153	Oct. 1967	Nov. 1967	HAND TITER VARIANCE	Dr. Rymer (Dr. Tripp)

TABLE I - Continued

SUMMARY OF STATISTICAL ANALYSES FOR FISCAL YEAR 1968

S.A.N.	DATE INITIATED	DATE COMPLETED	TITLE	RESEARCHER
154	Oct. 1967	Oct. 1967	RESPONSE CRITERION	Dr. Montes de Oca
155	Oct. 1967	Oct. 1967	GEOMETRIC MEANS	Dr. Baer
156	Oct. 1967		MULTIPLE IMMUNOGENS	Dr. Chaparas
157	Oct. 1967	Oct. 1967	COMPARISON OF BURKITT AND HERPES ANTIBODIES (INFANTS)	Dr. Gerber
158	Oct. 1967	Oct. 1967	RETROSPECTIVE STUDY ON BURKITT ANTIBODIES	Dr. Gerber
159	Oct. 1967	Oct. 1967	GEOMETRIC MEANS (INFANTS), PART II	Dr. Gerber
160	Oct. 1967		TUBERCULIN ANALYSIS	Mr. Kolb
161	July 1967	Nov. 1967	TUBERCULIN STABILITY, PART II	Dr. Baer
162	Nov. 1967	Suspended	PPD TUBERCULIN TINE TEST	Dr. Baer
163	Dec. 1967	Jan. 1968	ONCOGENESIS SCREENING	Mrs. Cox
164	Dec. 1967	Mar. 1968	VIRUS COUNT	Dr. Hobbins
165	Dec. 1967		CATECHOL VARIANCE	Dr. Baer
166	Jan. 1968	Jan. 1968	VIRUS AFFINITY	Dr. Gerber
167	Jan. 1968	Ref. S.A.N. 171	TINE-MANTOUX COMPARISON	Dr. Baer
168	Jan. 1968	Feb. 1968	HISTAMINE RESPONSE, PART II	Dr. Baer

TABLE I - Continued

SUMMARY OF STATISTICAL ANALYSES FOR FISCAL YEAR 1968

S.A.N.	DATE INITIATED	DATE COMPLETED	TITLE	RESEARCHER
169	Jan. 1968	Mar. 1968	ANTIBODY REACTIVITY	Dr. Chaparas
170	Jan. 1968	Jan. 1968	CORRELATION COEFFICIENTS	Dr. Uhlendorf
171	Jan. 1968		TINE EFFECTIVENESS	Dr. Baer
172	Jan. 1968	Feb. 1968	ALLERGENIC CHARACTERISTICS	Dr. Baer
173	Jan. 1968		DISEASE SEVERITY INDEX	Dr. Maloney
174	Jan. 1968		EXACT 3 X 3 CONTINGENCY TABLE PROBABILITY	Dr. Maloney
175	Mar. 1968		PPD RESUBMISSION	Dr. Baer

*Not included in FY 1967 Report.

Serial No. DBS 111
1. Office of the Director
2. Biometrics Section, DBS
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Development of Statistical Methodology Applicable to Biologics Control.

Previous Serial Number: DBS 111.

Principal Investigator: Clifford J. Maloney, Ph.D.

Other Investigators: Harold Baer, Robert W. Kolb, S. D. Chaparas, and George Shakarji.

Cooperating Units: Laboratory of Bacterial Products, DBS, Laboratory of Control Activities, DBS, and Computation and Data Processing Branch, DCRT.

Man Years:

Total:	0.6
Professional:	0.5
Other:	0.1

Project Description:

Objective: As need arises from the research activity of the Division for statistical technique not adequately treated in the literature and when it appears that a need for such technique will continue on an appreciable scale in the future, theoretical and computational work is undertaken to develop appropriate technical tools.

Subproject 1: A comprehensive evaluation of the official protocol for assaying potency of tuberculin has been in progress for some time.

Subproject 2: A more satisfactory evaluation of histamine sensitivity by a skin test is desired. A small trial to investigate the role of volume delivered, care in inoculation, age and sensitivity of patient, choice between wheal or erythema measurement and between using mean diameter or cross diameter products was analyzed.

Subproject 3: The logical structure of the cross reaction of different tuberculins based on homologous and heterologous reactions was investigated.

Subproject 4: Factors affecting the incidence of false negative and false positive reactions to Tine testing using PPD for tuberculin testing was available in nearly 18,000 records of comparative tests.

Methods:

Subproject 1: Conventional statistical methods are being applied by computer to a large body of experience obtained by the Division in routine control testing of tuberculins.

Subproject 2: Conventional statistical methods were applied to a small experiment to discriminate the best way to utilize readings from routine skin testing in medical practice.

Subproject 3: A technique of ordering the rows and columns of a matrix of observations subject to experimental error, previously developed by the Principal Investigator, was adapted to the analysis of results from heterologous delayed sensitivity skin reactions.

Subproject 4: Contingency table analysis was applied to a body of some 18,000 tuberculin skin test results to reveal the essential factors influencing the appearance of false positive and false negative findings.

Findings:

Subproject 1: The standard protocol in current use was verified as nearly if not fully as good as a more elaborate analysis based on calculation by least squares. A summary of the results to date was presented at a Conference on Evaluation of Procedures for Tuberculin Testing in February 1968. More recent data has been transmitted to the Computer Division for analysis. The results, when obtained, will be prepared for publication.

Subproject 2: Use of cross diameter sums (CPDs) of erythema gave somewhat better results than 3 other possible criteria in a small trial. Further progress depends on research of additional data.

Subproject 3: With some anomalies it proved possible to order 6 tuberculins in a rank order interpreted as representing the complexity of antigens present in the several species.

Subproject 4: Strikingly different incidence of false negative and false positive results were obtained in Tine PPD testing by different investigators. These latter were substantially influenced by the choice of induration size recognized as positive. A continuous rise in percent of positive reactors in the population with age was demonstrated.

Significance to Bio-Medical Research and the Program of the Division:

The current studies verified the adequacy of the current tuberculin testing procedure, throw light on the choice of most satisfactory

response metameter, and provide a new application for a ranking procedure which should prove useful in further studies with tuberculin antigens.

Publications: Baer, Harold, Kolb, Robert W., and Maloney, Clifford J.: The Standardization of Tuberculin, OT and PPD, by the Division of Biologics Standards, Transcript of Proceedings, Conference on Evaluation of Procedures for Tuberculin Testing, Bethesda, Maryland, February 1968.

Maloney, C. J., Bryan, S., and Epstein, M.: Computer Assisted Primary Index Preparation, J. of Chemical Documentation, Vol. 7, No. 4, Nov. 1967, pp. 223-232.

Annual Report of the Office of Investigational New Drugs
Division of Biologics Standards
July 1, 1967 - June 30, 1968

This office has the continuing responsibility for the receipt and evaluation of "Notices of Claimed Investigational Exemption" for new biological products.

During the period covered by this report 61 "Notices" were received. This brought to 305 the total number of "Notices" received since the Food and Drug Regulations requiring submissions became effective. Disposition of "Notices" received during the period 1963-1968 is shown in the following table:

Active	196
Withdrawn	108
Forwarded to FDA for processing	1
Total	305

The number of supplements received on active IND's throughout the year depends on the activity and number of investigators involved in the respective clinical trials. Following initial review of the "Notices", this office has the responsibility for informing sponsors of deficiencies in the submissions. Liaison with the sponsors and the Food and Drug Administration is maintained.

The information contained in these "Notices" may indicate to the scientific staff of the Division the paths in which research should be directed for the purpose of developing Regulations for monitoring the safety and potency of new biological products. Sponsors of IND's are encouraged to submit all available data for review so that judgments as to the safety and potency can be made well in advance of the time of licensing.

The members of this office participate actively in the scientific life of the DBS and of the National Institutes of Health attending conferences and seminars as well as representing the Division in a liaison capacity with appropriate committees of other institutes. Conferences are held periodically with representatives of manufacturers and others interested in investigative new drugs.

Annual Report of the Contract Operations Office
Division of Biologics Standards
July 1, 1967 through June 30, 1968

During the past three years a contracts program established to serve as an extension of, and supplement to, the Division's research and control activities, has undergone rapid, but carefully controlled growth.

The Division presently supports twenty-two active contracts, the majority of which are directly related to questions involving oncogenesis of viral vaccines and carcinogenic or toxic properties of vaccine components, adjuvants or other Biologics.

It is anticipated that the present level of contract activities will remain relatively constant as project terminations balance new project needs.

Details of all individual contractual projects will be found in the Contract Narratives following each Laboratory Chiefs' summary, in those instances where Project Officers are assigned from the individual laboratories to manage the projects. Other projects, initiated and managed by the Contracts Staff, are included in the section, Adjuncts to the Office of the Director. All contracts are included in a special index to this report.

Also, through the contractual mechanism, the Division held a "Conference on Cell Cultures for Virus Vaccine Production" at the Clinical Center, National Institutes of Health, in November, 1967. Dr. Donald Merchant of the University of Michigan was the organizer and chairman. The papers presented indicated the great need for controlled, well-studied, uncontaminated cell cultures capable of supporting the growth of a wide spectrum of viruses. Such cultures must be free from adventitious agents and have a diploid chromosome number. The proceedings of this conference will be published, as a monograph, by the Journal of the National Cancer Institute.

Although the contracts staff personnel participate extensively in the supervision of contractual operations, providing materials for tests and examining results of day-to-day activities, the scientific staff of the Division has contributed greatly to the development and function of the contracts program. This contribution has been of great value in the initiation, the supervision and in the evaluation of individual contracts as well as the overall contracts program. This has involved considerable expenditure of time on the part of individual scientists, often made at the sacrifice to their own intramural and collaborative research programs. In addition, some of the contracts staff are engaged in their own individual research projects.

CONTRACT NARRATIVES
DIVISION OF BIOLOGICS STANDARDS
July 1, 1967 through June 30, 1968

UNIVERSITY OF MICHIGAN (PH43-67-1154)

Title: Conduct conference on cell cultures

Contractor's Project Director: Dr. Donald J. Merchant

Project Officer (DBS): Dr. Roderick Murray

Objectives: To conduct a three-day conference for the purpose of assemblying available knowledge of the disciplines involved in cell culture systems and to review experience with virus vaccines.

Methods Employed: The three-day meeting was divided into five sessions: the first two were devoted to characteristics of cell culture systems; the third, neoplastic properties of animal cell lines; the fourth, viral oncogenicity and viral vaccines; and the fifth, experience with viral vaccines. The sessions were chaired respectively by Drs. V. J. Evans, Tissue Culture Laboratory, National Cancer Institute; M. M. Sigel, Chairman, Department of Microbiology, University of Miami; Jacob Furth, Professor, Department of Pathology, College of Physicians and Surgeons of Columbia University; Carl Habel, Scripps Clinic and Research Foundation; and H. Koprowski, of the Wistar Institute. Participants included some 90 leading virologists and cell culture specialists representing many countries throughout the world.

Major Findings: The proceedings of this conference will be published in the Journal of the National Cancer Institute.

Significance to DBS Program and Biomedical Research: This conference has enabled the Division to more clearly define problem areas in cell culture systems presently in use and to consider the possible use of other cell systems in the production of vaccines.

Proposed Course of Project: This contract is scheduled for termination on May 31, 1968.

Date Contract Initiated: June 1, 1967

Current Annual Level: $27,460

IIT RESEARCH INSTITUTE (PH43-67-1106)

Title: Studies of Latent Infection: Tissue Culture Phase

Contractor's Project Director: Dr. H. M. Yamashiroya

Project Officer (DBS): Dr. B. G. Young (Latent Virus) and Dr. M. F. Barile (Mycoplasma)

Objectives: To investigate, by tissue culture methods, primary African green monkey kidney, canine kidney, duck embryo and a continuous line of human lung fibroblasts (WI-38 cells) for covert contaminating viruses and Mycoplasma.

Methods Employed: Primary cultures of the above cells and continuous lines of WI-38 cells have been maintained in tissue culture for a minimum of sixty (60) days. Aliquots of the cultured cells are being disrupted by freezing and thawing and the cell lysates are subjected to centrifugation procedures designed to concentrate any virus present. The final centrifugation is into agar pellets which are being inoculated onto primary monolayers of human embryonic kidney, canine kidney, African green monkey kidney, duck embryo and onto a continuous line of WI-38 cells. These monolayers are being further passaged for 15 passages and observed microscopically for cytopathic or other viral effects. Agents revealed by this procedure are being passaged to high titer, serologically classified and subjected to neutralization attempts with the donor animals serum in order to demonstrate initial presence. When indicated, these agents will be furnished to an animal phase contractor for *in vivo* studies.

Major Findings: Preliminary results of this contract indicate that a large number of cytopathic viral agents are being revealed by the technical approaches employed. Mycoplasma also is being isolated from each cell species tested. Considerable information is also being accumulated with respect to the incidence of intestional and blood parasites infecting the donor animals.

Significance to DBS Program and Bio-Medical Research: This is one of a series of approaches designed to determine the presence or absence of covert viral agents and of Mycoplasma in cells used for vaccine virus production or in cells proposed for use in vaccine virus production. Results of this and related studies should allow the Division to make recommendations relating to the use of cell substrates for vaccine production.

Proposed Course of Project: This contract is currently scheduled for a one year period which began May 23, 1967, and is scheduled for termination July 22, 1968. Consideration is being given to the extension of this

contract to allow study, in a similar fashion, of other tissues used or proposed for use as vaccine substrates.

Date Contract Initiated: May 23, 1967.

Current Annual Level: $99,477 for 14 months.

MELPAR, INCORPORATED (PH43-67-1100)

Title: Studies of Latent Infections: Tissue Culture and Animal Phases

Contractor's Project Director: Dr. John Verna

Project Officer (DBS): Dr. B. G. Young (Latent Viruses) and Dr. M. F. Barile (Mycoplasma)

Objectives: To investigate primary African green monkey kidney, canine kidney, duck embryo and a continuous line of human lung fibroblasts (WI-38 cells) for covert oncogenic and/or other adventitious covert viruses and for Mycoplasma infection.

Methods Employed: Primary cultures of the above cells and a continuous line of WI-38 cells have been maintained in tissue culture for a minimum of 60 days. Aliquots of the cultured cells have been inoculated subcutaneously into newborn homologous animals as well as into Syrian hamsters and BALB/c mice. These animals are being maintained and continuously monitored for tumors and/or other pathogenic responses.

Other aliquots of the cultured cells are being planted onto primary monolayers of human embryonic kidney, African green monkey kidney, duck embryo, rabbit kidney, mouse embryo and upon WI-38 cells. These feeder layers are being observed for a minimum of twenty-one (21) days for cytopathic or other effects. Agents thus revealed are being serologically classified and grown to high titer and, when indicated, will be inoculated into newborn homologous and heterologous animals.

Two tissues from African green monkey kidney donors and canine kidney donor (laryngeal and urethral), as well as all primary cell monolayers, are being screened for the presence of Mycoplasma. This screening utilizes two incubation conditions (aerobic and anerobic) and two different mediums. Mycoplasma revealed in this procedure are prepared to high titer and maintained for serologic identification.

Major Findings: It is apparent that the substrates examined in this contract harbor numerous adventitious agents, both viruses and Mycoplasma. Insufficient time has elapsed for conclusions to be drawn relative to the oncogenic potential of any of these revealed agents.

Significance to DBS Program and Bio-Medical Research: This is one of a series of experimental approaches designed to determine the presence or absence of covert oncogenic and/or other adventitious microbial agents in cells used for vaccine virus production or in cells proposed for use in vaccine virus production. Results of this and related studies should allow the Division to evaluate the relative hazards of vaccine substrates and to

make recommendations relating to the use of cell substrates for vaccine production.

Proposed Course of Project: This contract, which is scheduled for a two year period, began May 24, 1967, and will terminate May 23, 1969.

Date Contract Initiated: May 24, 1967.

Current Annual Level: $229,441.

YALE UNIVERSITY (PH43-67-1463)

Title: A Statistical Approach to Human Karyotype

Contractor's Project Director: Dr. Frank H. Ruddle

Project Officer (DBS): Dr. B. G. Young

Objectives: The purposes of this contract are to determine the precise definition of the chromosome constitution of man and to seek the solution of certain problems relating to the stability of cultured human cells.

Methods Employed: Lymphocyte cultures are being established from the cord blood of approximately 4500 human newborns and approximately 1000 of their mothers. Slides of metaphase chromosomes are prepared from these cultures and photographed. Idiograms are made from the phtomicrographs and the chromosome arm length measurements are determined. Semi-automated and fully-automated scanning devices are also employed to supplement the conventional manual measurements. Primary diploid human cells in tissue culture (WI-38 cells) are also being examined to determine a correlation, if any, between karyotype and growth kinetics, mean cell volume, and ageing of the cells. Clinical coding of the data has been designed to permit direct punching of the data onto IBM cards. This data is then processed by computer.

Major Findings: To date approximately 2300 lymphocyte cultures have been initiated from human infants. Clinical data has been obtained for approximately 2200 cultures. Conventional manual measurements have been completed on approximately 1800 cultures (2 cells per culture) and computer analysis of the first 1000 photomicrographs has been accomplished. Early results indicate approximately 0.5 percent of the chromosome complements will be grossly abnormal, for example, YY, XXX, translocations, trisomics, XXXY mosaics, and approximately 0.05 percent of the infants phenotypically abnormal. Thus far, less drastic deviations from the average karyotype approximate 25 percent.

Preliminary results obtained with the human lung fibroblastic line (WI-38 cells) indicate that these cells have a finite life expectancy of approximately 50 cell doublings. With increasing passage number, the instantaneous growth rate constant progressively decreases accompanied by a shortening of the lag period; and an entrance into plateau phase of growth at lower cell densities. Until the fiftieth doubling there occurs a constant increase in the mean cell volume accompanied by an increase in cells of higher ploidy values. The increase in cell volume may be related to, but not totally accounted for, by the increase of tetraploid cells. It should be emphasized that these are preliminary observations which remain to be confirmed.

Significance to DBS Program and Bio-Medical Research: This contract has two primary objectives. The first objective is to establish a reliable definition of the chromosome constitution of man and the second objective is the solution of certain problems relating to the stability of continuous human cell lines *in vitro*.

Insufficient data presently exists to reliably establish the normal chromosome constitution of man and the frequency of normal and abnormal variants. A precise definition of the chromosome constitution of man and a better understanding of the events associated with senescence in cultured human cells might allow the Division to make recommendations concerning the use of human cells in vaccine production and may lead to new tissue culture techniques for evaluating the safety of Biologics.

Proposed Course of Project: Work under this contract was initiated June 29, 1967, and is scheduled for termination December 28, 1968. However, the scope of this work is such that two such contract periods might be necessary to realize the full benefit of the study.

Date Contract Initiated: June 29, 1967.

Current Annual Level: $297,746 for 18 months.

WOODARD RESEARCH CORPORATION (PH43-67-1397)

Title: Investigation of the Reaction Products of β-Propiolactone

Contractor's Project Director: Dr. D. S. Bates

Project Officer (DBS): Dr. B. G. Young

Objectives: This contractor is investigating the reaction of beta-propiolactone with a variety of biological materials in order to determine the nature of the products of the reaction and any affinity of beta-propiolactone for a particular substrate. Biological properties of beta-propiolactone and its reaction products are being determined by subcutaneous injection into weanling and newborn CAF_1 mice.

Methods Employed: Reaction products are separated by chromatographic procedures which employ ninhydrin or ultraviolet visualization to demonstrate their location. Results are confirmed and extended using beta-propiolactone-H^3 and radioautography. Weanling CAF_1 mice have been injected subcutaneously with beta-propiolactone and with the reaction products of beta-propiolactone and ten (10) selected biological materials to determine the presence or absence of oncogenic properties.

Major Findings: To date, R_f values have been determined for twenty-six (26) of thirty-six (36) biological test compounds. Reaction products have been detected in most instances. All mouse inoculations have been completed and the inoculated mice are being monitored for tumor production. No tumors have as yet been observed.

Significance to DBS Program and Bio-Medical Research: Beta-propiolactone is used as a virus inactivating agent in vaccine production. During virus inactivation beta-propiolactone is reported to be completely hydrolyzed to innocuous end products. Non-hydrolyzed beta-propiolactone has been reported to be mutagenic in Neurospora and oncogenic when applied to the skin of mice. This contract should demonstrate whether, during hydrolysis, beta-propiolactone can impart oncogenic properties to non-oncogenic biological compounds. This knowledge will allow the Division to make recommendations on the continued use of beta-propiolactone as a virus inactivating agent.

Proposed Course of Project: Work under this contract commenced June 28, 1967, and is expected to be completed June 27, 1969.

Date Contract Initiated: June 28, 1967.

Current Annual Level: $99,290 for 24 months.

CONTRACT NARRATIVES
DIVISION OF BIOLOGICS STANDARDS
July 1, 1967 through June 30, 1968

BIO-RESEARCH CONSULTANTS (PH43-67-677)

Title: Study of Carcinogenic Potential of 7 Chemicals Used in Biological Preparations

Contractor's Project Director: Dr. Freddy Homburger
Dr. Peter Bernfeld

Project Officer (DBS): Dr. Stanley H. Singer

Objectives: To test the oncogenic potential and co-carcinogenic activity of the following 7 compounds found in biological products as preservatives, inactivating agents, stabilizers, or additives: thimerosal, benzethonium chloride, parabens, phenol red, pyridine, ethylene glycol, and ethylene chlorohydrin.

Methods Employed: Methods designed to accelerate and enhance detection of any carcinogenic potential include: pooling of injection sites with reintroduction into secondary homologous hosts, the use of a sensitive indicator animal, and co-carcinogen studies.

Major Findings: To date there has been no indication of any carcinogenic potential of any of the seven test compounds in this study.

Significance to DBS Program and Bio-Medical Research: These programs constitute part of the continuing effort to assure that various components of biological products are free from carcinogenic or co-carcinogenic activity.

Proposed Course of Project: This contract will terminate August, 1968.

Date Contract Initiated: May 23, 1967.

Current Annual Level: $32,000.

UNIVERSITY OF MARYLAND (PH43-68-646)

Title: Study of Recipients of Attenuated Measles Virus Vaccine Prepared in Canine Kidney Cells

Contractor's Project Officer: Dr. Fred R. McCrumb, Jr.

Project Officer (DBS): Dr. Stanley H. Singer

Objectives: This study will review the health status of approximately 8,000 recipients of attenuated measles-virus vaccine prepared in canine kidney tissue culture cells.

Methods Employed: Data will be collected from the State of Maryland and the municipality of St. Joseph, Missouri, by direct contact with physicians and household heads.

Major Findings: None. This study has only recently been initiated.

Significance to DBS Program and Bio-Medical Research: This study is part of the continuing effort of monitoring the safety of vaccines.

Proposed Course of Project: This contract is anticipated to be completed within a twelve (12) month period.

Date Contract Initiated: February 28, 1968.

Current Annual Level: $33,000.

CONTRACT NARRATIVES
DIVISION OF BIOLOGICS STANDARDS
July 1, 1967 through June 30, 1968

UNIVERSITY OF PITTSBURGH (PH43-67-701)
UNIVERSITY OF MINNESOTA (PH43-67-705)

Title: Studies of Patients Receiving Exchange Transfusions in Neonatal Period

Contractor's Project Director: Dr. Edward R. Schlesinger, University of Pittsburgh
Dr. Allyn G. Bridge, University of Minnesota

Project Officer (DBS): Dr. Stanley H. Singer

Objectives: These contracts will investigate whether the administration of exchange transfusions which may contain extraneous agents, such as latent viruses, results in any diseases in later life over and above control populations which did not receive exchange transfusions. Particular attention will be given to leukemia and other diseases of possible viral etiology.

Methods Employed: A group of infants who received exchange transfusion and an appropriate control group have been identified via a search of hospital records. Health histories of these groups will be obtained via questionnaire or personal interview of parents and, in some cases, physicians.

Major Findings: Insufficient time has elapsed for collection of enough data to be meaningful.

Significance to DBS Program and Bio-Medical Research: This program will help the Division to fully discharge its responsibilities for the safety of blood and blood products. In addition, although exchange transfusion is a common occurrence in current medical practice, there is a dearth of studies available in the literature which indicate the health status of these children several years after transfusion. This study will help fill this gap.

Proposed Course of Project: Contract with the University of Pittsburgh began May 1, 1967, and will continue for a total of twenty-four months. Contract with the University of Minnesota was initiated April 7, 1967, and was renewed April 1, 1968, for an additional twelve (12) month period.

Contract Level: University of Pittsburgh - $119,687 for 24 months
University of Minnesota - $71,000 for second year's effort.

BIONETICS RESEARCH LABORATORIES (PH43-67-1483)

Title: Studies of the Oncogenic Activity of Human Biological Products in Syrian Golden Hamsters (Mesocricetus auratus)

Contractor's Project Director: Dr. John Landon

Project Officer (DBS): Dr. Stanley H. Singer

Objectives: To study human biological products for oncogenic activity when inoculated into newborn Syrian golden hamsters.

Methods Employed: The products to be studied are purchased from commercial sources in their final containers. These compounds are coded by DBS staff members and delivered to the contractor where they are inoculated into newborn hamsters within 24 hours after birth by a single subcutaneous inoculation. These animals are observed daily, weaned at 21 days, divided into groups of 8 or less animals and observed closely for the development of palpable masses or other indications of neoplasitc disease. All animals which die are subjected to thorough histopathologic examination except when cannibalism or autolysis will not permit. Transplants to weanling hamsters are attempted on all test animals which develop palpable masses. Blood serum and portions of the neoplasm are retained at -70°C. for further studies. Positive and negative controls are included in the study.

Major Findings: Animals inoculated with positive controls have developed neoplasms with high incidence and with proper latent periods. There has been a low incidence of tumor formation scattered amongst the remaining groups which is thought to represent spontaneous tumor formation in the colony.

Significance to DBS Program and Bio-Medical Research: This is one approach to determine the safety of available human biological products. Although oncogenic activity in hamsters cannot presently be related to humans, results from hamster studies should shed some light on safety of these products.

Proposed Course of Project: This project is scheduled for termination in fiscal year 1970.

Date Contract Initiated: July 1, 1966

Current Annual Level: $303,260

Serial No. DBS 132
1. Office of the Director
2. Contract Operations Office
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of virus-cell interactions

Previous Serial Number: DBS 132

Principal Investigator: B. G. Young, Ph.D.

Other Investigators: B. E. Engels, B.A.

Cooperating Units: Laboratory of Bacterial Products and Laboratory of Pathology

Man Years
Total: 2.50
Professional: 1.50
Other: 1.00

Project Description:

Objectives: To isolate and characterize the "herpes-like" virus present in cultured human lymphoblasts. Special attention is being given to the immunologic character of the infected cell, the reactions of this cell with "normal" globulins of human origin and the interferon content of nine of these continuous cell lines. Correlations between virus content, positive indirect immunofluorescence, sensitivity to a cytotoxic reaction and interferon content are being established. Also under investigation, in collaboration with the DBS Laboratories of Pathology and Bacterial Products, are the potential usefulness of tissue cultured insect cells (Aedes aegypti) as virus substrate and the effect of Mycoplasma contaminants on the above-mentioned experimental parameters.

Methods Employed: Virus infectivity titrations in tissue cultures, quantitation of interferon production and fluorescence microscopy are some of the methods used to study selected virus-cell systems.

Major Findings: Pools of normal human immune globulins contain substances which suppress growth of cells derived from malignant tissues. This suppression does not occur with cells derived from "normal" human embryonic tissue or with cells derived from other species (chimpanzee). The suppression is not complement dependent and is more pronounced with some continuous lines than with others. Such pools also contain substances which react with these cells to yield a positive indirect immunofluorescence

test. Different cellular antigens are involved as demonstrated by results following mild trypsinization treatment and by adsorption procedures utilizing homologous and heterologous cells.

With one exception, each continuous human line has been shown to contain a material possessing the properties of interferon. The titer of this interferon varies widely between cell lines.

Significance to Bio-medical Research and the Program of the Division: The etiologic significance of the "herpes-like" virus ubiquitously present in cultured human lymphoma and leukemia cells has yet to be evaluated. A demonstration of the presence of cytotoxic antibody and the frequency of this occurrence in "normal" pools of human globulins should contribute to an assessment of the etiologic significance of the virus. Likewise, a determination of the quantity of interferon present in continuous lines correlated, if possible, with the presence or absence of virus should also contribute to an evaluation of the etiologic significance of the virus. An evaluation of the role of Mycoplasma in suppressing or enhancing the experimental responses of the cell also adds to an understanding of the mechanisms involved in such reactions.

Proposed Course of Project: These studies will continue in the coming year.

Honors and Awards: None

Publication: Nadel, E.M., Banfield, W.G., and Young, B.G.: Virus Particles Visualized by Electron Microscopy in Strain 2 Guinea Pig Leukemia L_2C/NB. Acta Cytologica 11: 347, 1967.

Serial No. 131
1. Office of the Director
2. Contract Operations Office
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Interferon Production by Oncogenic Viruses

Previous Serial Number: 131

Principal Investigator: Stanley H. Singer, M.D.

Other Investigators: Ruth L. Kirschstein, M.D.
S. Baron, M.D.

Cooperating Units: Laboratory of Pathology, DBS
Laboratory of Viral Diseases,
Section on Cellular Virology, NIAID

Man Years:

Total:	1.6
Professional:	0.7
Other:	0.9

Project Description:

Objectives: The differences between adenoviruses oncogenic for hamsters and adenoviruses not oncogenic for hamsters are being investigated. The effects of these viruses on cellular biology with particular emphasis on production of interferon or other viral inhibitors is being studied.

Methods: Hamster cells are established in tissue culture and are then infected with a selected adenovirus type. The effects of the adenovirus on the cell are then measured by various methods, including superinfection with challenge viruses, testing of supernatants for biologic activity, and measurement of RNA and protein synthesis.

Major Findings: Interferon formation or the lack of it does not appear to be related to the oncogenicity of a virus. Adenovirus 5, a virus not oncogenic for hamsters, has been found to induce an intracellular viral inhibitor which does not appear to be interferon mediated. A stock preparation of adenovirus 12, a virus oncogenic for hamsters, has been noted to induce a viral enhancer.

Significance to Bio-Medical Research and the Program of the Division: These studies may provide additional information on those factors necessary for a virus to be oncogenic. In addition, interferon being developed may be of use in evaluating adenoviruses which may be potential vaccine candidates.

Proposed Course: These studies will continue in the coming year.

Honors and Awards: None

Publications: None

Annual Report of the Information Office
Division of Biologics Standards
July 1, 1967 - June 30, 1968

The DBS Information Office carried out an active public information program this year, not only through assistance to the media in response to its many requests, but in making available general information regarding the Division.

National magazine features: The American Professional Pharmacist's July issue published an 8-page cover story on the DBS, by-lined by the Director, and titled "Biologics Old or New: Research is Vital." The August issue of Hospital Formulary Management published a 3-page cover story titled "DBS: Small Agency with a Big Job." The Medical World News, December 8 issue, featured the DBS in a 5-page story with 4-color cover photo of Director. JAMA and Medical World News carried stories on DBS research in March. The JAMA covered the two-day tuberculin symposium, sponsored by DBS and the American Thoractic Society; MWN covered DBS's work on the development, standardization, and licensing of coral snake antivenin. The Information Office arranged for the coverage, and interviews for both stories, and supplied background and photos.

Brochure: A 16-page brochure on the Division, prepared by the Information Office, was published in March. Photos illustrate animal tests, laboratory techniques, and research-related control and test procedures, all a part of DBS's responsibility for maintaining standards of quality and safety. Over 3,000 requests for the brochure had been filled by May 1, following the initial distribution of 3,500 in mid-March. Requests came from state health departments, hospitals, medical schools and libraries, foreign and domestic pharmaceutical manufacturers, voluntary health organizations, government and independent research organizations.

Press, TV and radio: Subjects eliciting the greatest number of queries from press, TV, and radio, were 1) influenza and reasons for apparent shortage of vaccine in face of anticipated epidemic; 2) a continuing and increasing interest in the status of rubella vaccine research; 3) the licensing of the first live mumps vaccine in December; 4) the long-heralded licensing of Rho immunoglobulin in April; and 5) the DBS embargo, finally lifted, on the use of African green monkeys by licensed vaccine manufacturers.

Press conferences: One was arranged in October on rubella for the American Academy of Pediatrics meeting in Washington. E. Mead Johnson Award winners Drs. Meyer and Parkman briefed 20 science writers from wire services and leading U.S. newspapers on the status of rubella vaccine research.

During the 3-day international conference on Cell Cultures for Virus Vaccine Production, sponsored by the DBS, a press room was maintained in the Clinical Center and daily press briefings were arranged by the Information Office.

Recurring assignments: NIH 1968-67 Directory-Bibliography; HEW Annual Report; NIH 1967 Almanac and DBS Who's Who revisions; research highlights and special report on rubella for 1969 appropriations hearings.

Annual Report of the Office of Scientific Communications
Division of Biologics Standards
July 1, 1967 through June 30, 1968

The responsibilities of the DBS Scientific and Technical Communications Office (OSC) are to plan, organize and implement appropriate programs for the collection, evaluation, storage, retrieval, and distribution of the scientific and technical data and information needed for the control and research programs of the Division.

The implementation, this year, of a rapid literature searching service (contract No. PH43-67-74) made it possible for the OSC to handle over 150 specific, and often complex, search requests and to offer the staff over 8,000 individual abstracts in response to specific technical problems.

A bibliography, containing 1,447 abstracts covering the world's research on cell cultures for virus vaccine production, was prepared for the Cell Culture Conference in November, 1967. This abstract bibliography was followed by a citation bibliography containing over 2,000 references.

The in depth indexing, of the World Health Organization working documents of the Expert Committee on Biological Standardization from 1947 to date, was completed this year. The completed master file of 10,000 cards was duplicated and the duplicate set sent to the WHO headquarters in Geneva, Switzerland. The completion of this project made possible the compilation of the first two parts of a five part DBS thesaurus.

Automation and indexing of the Central Files, mentioned last year, has been postponed until 1969, due to lack of funds.

The DBS library and reference room was organized for more effective staff use.

The informal network for sources of information continues to grow each year and now includes not only other Divisions, Institutes, Federal agencies, private industries, societies and libraries but many individual scientists in the private and university sectors. Over 300 different individual sources for information were consulted during this fiscal year by the OSC to meet Division problems and needs.

Routinely the OSC assembles and indexes the Division's Annual Report each year.

The utilization of the most effective procedures for meeting the scientific and technical information needs of the Division will continue to be the main objective of the office. However, limited funds and personnel make long-range planning difficult.

Contract Narrative
Office of Scientific Communications
Division of Biologics Standards
July 1, 1967 through June 30, 1968

BIOLOGICAL ABSTRACTS, INC. (PH43-67-74)

Title: A literature searching and abstracting program to meet the specific needs of the Division of Biologics Standards.

Contractor's Project Director: No project officer was appointed by the contractor.

Project Officer (DBS): Mrs. A. K. Reich

Objectives: The goal of the program was to give comprehensive and timely coverage of the biomedical and biological literature, as related to biological products, to meet the specific needs of the DBS research and contracts program.

Methods Employed: A direct-line on-demand search service, using an electrowriter-dataphone hook-up between DBS and Biological Abstracts, was used to receive scientific and technical information covering over 7,000 journals in the biological sciences, in response to specific questions asked by the DBS scientific staff. All questions were a direct result of problems to be solved within the Division to meet responsibilities to the public, and covered all aspects of the scientific, technical, control, and regulatory activities. Both "single" and "standing" requests were handled. The output of the search service was a microfilmed reproduction of each author abstract with complete citation.

Major Findings: The program gave the Division a new service which filled many needs. However, lack of cover-to-cover indexing by the contractor and the 24 day service did not meet many Division demands. This first year pilot study made conspicuously clear the fact that the DBS must have a much more timely and multidisciplined informational source.

Significance to DBS Program & Biomedical Research: The service gave much needed support to the basic research and contracts programs of the Division inspite of its many shortcomings.

Proposed Course of Project: The contract is being renegotiated with a new contractor in order to supply the Division with better biomedical and clinical coverage, and to give more timely service. The program is expected to expand as needs increase.

Date Contract Initiated: March 16, 1967

Date Present Contract Terminated: March 15, 1968

Current Annual Level: Funding for second year is expected to be around $60,000

INDEXES

INDEX TO CONTRACTS
Division of Biologics Standards
July 1, 1967 through June 30, 1968

Number	Title	Page
PH43-64-76	Preparation of Poison Ivy and Poison Oak Extracts and the Synthesis of Substances Related to the Active Principles	9
PH43-64-885	Determination of Potency and Efficacy of Urushiol, the Active Ingredient of Poison Ivy Extract	11
PH43-65-1046 PH43-68-97	Effects of Storage Conditions on Plastic Blood Containers	79
PH43-66-552	Serotyping of *Bordetella pertussis* Cultures	13
PH43-66-1163	Preparation of Mycoplasma Antigens and Antisera	161
PH43-67-74	A Literature Searching and Abstracting Program	286
PH43-67-675	Investigations on the Mechanisms of Chemical Carcinogenesis	99
PH43-67-676 PH43-67-677 PH43-67-684	A Study of Carcinogenic Potential of Seven Chemicals Used in Biological Preparations	81, & 274
PH43-67-679	Studies on the Biologic Activity of Oil Adjuvants	98
PH43-67-701 PH43-67-705	Studies of Patients Receiving Exchange Transfusions in Neonatal Period	276
PH43-67-1100	Studies of Latent Infections: Tissue Culture and Animal Phases	269
PH43-67-1101	Chemical and Physical Analysis of Oils and Emulsifiers	101
PH43-67-1104	The Metabolic Fate of an Emulsion Containing Mineral Oil and Arlacel A Using Radioactive Carbon Tracers	100
PH43-67-1105	Electron Microscope Services	197
PH43-67-1106	Studies of Latent Infection: Tissue Culture Phase	267

Number	Title	Page
PH43-67-1154	Conference on Cell Cultures for Virus Vaccine Production	266
PH43-67-1397	Investigation of the Reaction Products of β-Propiolactone	273
PH43-67-1463	A Statistical Approach to Human Karyotype	271
PH43-67-1483	Studies of the Oncogenic Activity of Human Biological Products in Syrian Golden Hamsters *(Mesocricetus auratus)*	131, & 277
PH43-68-632	Determination of Potency and Efficacy of the Active Ingredient of Poison Oak Extract by Clinical Studies	15
PH43-68-646	Study of Recipients of Attenuated Measles Virus Vaccine Prepared in Canine Kidney Cells	275

INDEX TO INVESTIGATORS
Division of Biologics Standards
July 1, 1967 through June 30, 1968

DBS scientists plus doctorate level collaborators, NIH only.

Albrecht, Paul, M.D. . . . p. 200, 201, 202, *Introduction*

Allen, Anton M., D.V.M. . . . p. 136, 206, 208

Amstey, Marvin S., M.D. . . . p. 169, 185, 186, 187, 255

Aronson, David L., M.D. . . . p. 86

Aulisio, Calvin G. . . . p. 206, 215, 217, 219, 220, 221, 242

Aylor, Harry . . . p. 113, 120

Bacon, Edward, Ph.D. . . . p. 137

Baer, Harold, Ph.D. . . . p. 7, 9, 11, 15, 16, 17, 18, 21, 22, 23, 24, 26, 117, 118, 134, 253, 256, 257, 259, 261

Baker, James . . . p. 232

Barber, Donald . . . p. 141

Barile, Michael F., Ph.D. . . . p. 33, 37, 38, 39, 41, 42, 43, 44, 161, 238, 241, 267, *Introduction*

Barker, Lewellys, M.D. . . . p. 134, 135, 175, 179, 184

Baron, Samuel, M.D. . . . p. 232, 280

Bell, Joseph A., M.D. . . . p. 243, 244

Berkeley, William H. . . . p. 105

Berman, B. A., M.D. . . . p. 26

Birch, Suzette M. . . . p. 173, 174

Bowser, Robert, Ph.D. . . . p. 19

Brown, Eva R. . . . p. 163, 170, 179, 256

Brown, Paul, M.D. . . . p. 238

Brown, Thomas F. . . . p. 45, 54

Burruss, Harry W. . . . p. 91, 92

Butterly, Arlene . . . p. 283, *Introduction*

Canchola, Jose G., M.D. . . . p. 211, *Introduction*

Causey, Ottis, M.D. . . . p. 238

Chaparas, Sotiros D., Ph.D. . . . p. 7, 16, 17, 19, 21, 28, 30, 32, 134, 135, 253, 255, 256, 257, 258, 259, *Introduction*

Chretien, Paul, M.D. . . . p. 19

Clark, G. D. . . . p. 61, 64

Clark, Ronald, Ph.D. . . . p. 39, 49, 128, 132, 134, 137, 139, 143, 146, 200, *Introduction*

Coghill, George E. . . . p. 215

Cohen, Pinya, Ph.D. . . . p. 73, 98, 99, 100, 101, 105, 106, 139, 140, *Introduction*

Cox, Claire . . . p. 129, 257

Crouch, Madge L. . . . p. 79

David-Ferreira, Jose, M.D., Ph.D. . . . p. 238, 242

Davis, Robert, M.D. . . . p. 148

Dawson, John H. . . . p. 105, 106

Dayton, Ann L. . . . p. *Introduction*

Demeo, Anthony N., M.D. . . . p. 19

Dennis, Lewis H., M.D. . . . p. 206, 207

Douglas, Robert D. . . . p. 163, 170, 175, 181, 184

Dowdle, Walter, Ph.D. . . . p. 243

Draper, Lawrence R., Ph.D. . . . p. 19

Dunlap, Ruth, Ph.D. . . . p. 197, 211, 222

Eddy, Bernice E., Ph.D. . . . p. 246, 251, 252, *Introduction*

Ehrenreich, Barbara L. . . . p. 82

Engels, B. E. . . . p. 278

Ennis, Francis A., M.D. . . . p. 169, 170, 172, *Introduction*

Enterline, Joan C. . . . p. 163

Esposito, Vito M., Ph.D. . . . p. 51

Farber, Jane F. . . . p. 102, 103, 107, 109, 115, 122, 124

Feeley, John C., Ph.D. . . . p. 7, 13, 45, 48, 51

Finlayson, John S., Ph.D. . . . p. 82, 83, 86, 87, 88, 89, 90

Fitzgerald, Edward . . . p. 129

Ford, Marshall . . . p. 150

Flam, Brenda . . . p. 82

Francis, Thomas C., D.D.S. . . . p. 41, 42

Friedman, Robert M., M.D. . . . p. 30

Fuller, Vernon J., Ph.D. . . . p. 111, 113, 114

Gainsburg, Duane B., M.D. . . . p. 49

Gajdusek, Carleton, M.D. . . . p. 238, 243

Gallelli, Joseph, Ph.D. . . . p. 16

Gardner, Roberta, Ph.D. . . . p. 50, 139, 140

Gehle, W. D. . . . p. 66, 67, 68, 70

Gerber, Paul, Ph.D. . . . p. 173, 174, 182, 183, 255, 256, 257

Gibbs, Clarence J., Ph.D. . . . p. 238, 242

Gibson, Sam T., M.D. . . . p. 73, *Introduction*

Godfrey, Henry, M.D. . . . p. 9, 11, 15, 16, 17, 21, 22, 24, 255, 256

Grabowski, Marion W. . . . p. 43

Graykowski, Edward A., D.D.S., M.D. . . . p. 41, 42

Green, Otis L. . . . p. 120, *Introduction*

Hale, R., M.D. . . . p. 26

Hamilton, Judith P. . . . p. 163

Hansen, Carl T., Ph.D. . . . p. 57

Hardegree, M. Carolyn, M.D. . . . p. 33, 35, 37, 38, 39, 40, 59, 72, 98, 99, 100, 101, 134, 135, 136, 139, *Introduction*

Harrah, John D., M.D. . . . p. 19

Hedrick, Sally . . . p. 19, 32

Helprin, Jerome J., Ph.D. . . . p. 35, 59, 70

Hiatt, C. W., Ph.D. . . . p. *Introduction*

Hill, Donald E. . . . p. 24

Hobbins, Thomas E., M.D. . . . p. 66, 67, 70, 163, 169, 170, 255, 256, 257

Hopps, Hope E. . . . p. 136, 143, 145, 161, 163, 170, 175, 179, 181, 184

Hudson, Herman . . . p. 82, 83, 86, 90

Jacobs, Leon, Ph.D. . . . p. *Introduction*

Jahnes, William G. . . . p. 209, 210, 217, 218, 219

Johnson, Joyce . . . p. 137, 146

Johnston, Paul B., Ph.D. . . . p. 238, 241

King, Michael L. . . . p. 246

Kirschstein, Ruth L., M.D. . . . p. 30, 39, 40, 49, 66, 68, 70, 132, 134, 135, 143, 145, 148, 200, 202, 209, 213, 280, *Introduction*

Kisher, C. T. . . . p. 88

Knapp, Catherine . . . p. 82

Kolb, Robert W. . . . p. 18, 37, 111, 113, 114, 117, 118, 120, 255, 257, 259, 261, *Introduction*

Larson, H. Elliott, M.D. . . . p. 163

Leventhal, Brigid G., M.D. . . . p. 43

Lewis, John, M.D. . . . p. 148

Li, Chen Pien, M.D. . . . p. 246, 252

Lorenz, Douglas, Ph.D. . . . p. 150, *Introduction*

McGrath, Philip . . . p. 137

Maloney, Clifford J., Ph.D. . . . p. 22, 26, 118, 253, 255, 258, 259, 261, *Introduction*

Manclark, Charles R., Ph.D. . . . p. 49, 57, 137, *Introduction*

Mann, Bruce . . . p. 185, 187

Markley, Kehl, M.D. . . . p. 148

Marquina, Ruben . . . p. 111, 113

Marshall, Harry P. . . . p. 71

Marshall, James F. . . . p. 49, 57

Mason, Richard A., D.V.M., Ph.D. . . . p. 202, 224, 226, 228

Mealey, Edward H., Ph.D. . . . p. 73, *Introduction*

Meyer, Barbara C. . . . p. 163, 170, 175, 181, 184

Meyer, Harry M., Jr., M.D. . . . p. 50, 143, 145, 163, 168, 169, 170, 172, 175, 181, 184, 283, *Introduction*

Miller, Carl E., D.V.M. . . . p. 45, 54, *Introduction*

Miller, J. Richard . . . p. 71

Mitchell, Freddie . . . p. 217

Montes de Oca, Hector, M.D. . . . p. 66, 70, 72, 230, 234, 256, 257, *Introduction*

Morris, J. A., Ph.D. . . . p. 238, 241, 242, 243, 245, 255

Morrison, Eleanor G. . . . p. *Introduction*

Murray, Roderick, M.D. . . . *Introduction* p. 265, 266

Mushinski, J. Frederic, M.D. . . . p. 86, 89, 90

O'Malley, Joseph P., M.D. . . . p. 263, *Introduction*

Oppenheim, Joost J., M.D. . . . p. 41, 42

Orr, Henry, Ph.D. . . . p. 64, 217, 230, 232, 234, 236, *Introduction*

Ozer, Harvey, M.D. . . . p. 134, 135

Palmer, Amos E., D.V.M. . . . p. 35, 129, 132, 134, 136, 139, 141, 143, 148, 206, 208, 213, *Introduction*

Parker, John, Ph.D. . . . p. 238

Parkman, Paul, M.D. . . . p. 143, 145, 163, 168, 169, 256, 283, *Introduction*

Paul, Elizabeth B. . . . p. 82, 83

Patt, Judith K. . . . p. 222

Peckinpaugh, Robert, M.D. . . . p. 243

Pedreira, Frank A., M.D. . . . p. 204, 211, 213

Pellissier, Aime, M.D. . . . p. 238

Pennington, R. M. . . . p. 85

Peters, Elizabeth . . . p. 66, 68, 70, 134, 135, 136

Pisano, John J. . . . p. 86, 87

Pittman, Margaret, Ph.D. . . . p. 7, 13, 33, 37, 38, 45, 49, 50, 51, 57, *Introduction*

Potkay, Stephen . . . p. 82

Potter, Michael, M.D. . . . p. 89, 90

Powell, Donald L. . . . p. 81

Prescott, Benjamin, Ph.D. . . . p. 246

Probst, George P. . . . p. 232

Rabson, Alan, M.D. . . . p. 134, 135

Reich, A. K. . . . p. 285, 286, *Introduction*

Riggs, Donald B. . . . p. 43

Roberts, Charles O. . . . p. 45

Robinson, Clarence A. . . . p. 71

Roelands, Joan . . . p. 82, 83

Rogers, Joseph L. . . . p. 230, 232

Root, Richard K., M.D. . . . p. 16, 17

Rosenblum, Edith N. . . . p. 173, 174, 182

Rowe, Wallace P., M.D. . . . p. 238

Russell, Elmer C. . . . p. 120

Rusten, George . . . p. 132, 150

Saglam, Mehmet, M.D. . . . p. 243

Scheno, Robert C. . . . p. *Introduction*

Scherr, M., M.D. . . . p. 26

Schofield, Frank, M.D. . . . p. 33

Seligmann, Edward B. Jr., Ph.D. . . . p. 37, 102, 103, 104, 105, 106, 107, 109, 115, 120, 122, 124, 143, 209, 255, *Introduction*

Shaw, C. W. . . . p. 242, 245

Sheagren, John N., M.D. . . . p. 19

Shelokov, Alexis, M.D. . . . p. 136, 143, 198, 200, 202, 204, 205, 206, 208, 209, 210, 211, 213, 217, 220, 221, 222, 230, 236, *Introduction*

Singer, Stanley H., M.D. . . . p. 81, 129, 131, 274, 275, 276, 277, 280

Smadel, J. E., M.D. . . . p. 242

Smith, Kendall O., Ph.D. . . . p. 60, 61, 63, 64, 66, 70, 82, 127, 134, 135, 136, 143

Spector, Sheldon L., M.D. . . . p. 209, 210

Stang, Peter C. . . . p. 82

Stanley, Walter, Ph.D. . . . p. 137

Stevan, Lee J. . . . p. 104, 105

Stevens, David P., M.D. . . . p. 175, 179

Stocks, Naomi T. . . . p. 232

Strott, Mary C. . . . p. 82, 83, 84, 85

Swinney, W., M.D. . . . p. 26

Szwed, Clarence F. . . . p. 79, 82, 84

Takemoto, K. K. . . . p. 134, 135

Tauraso, N. M., M.D. . . . p. 66, 69, 136, 143, 204, 205, 206, 208, 209, 210, 211, 213, 215, 217, 219, 232

Taylor, Mervin . . . p. 86, 87

Thiel, J. F. . . . p. 60, 61, 63, 64, 66, 70

Tomar, Russell H., M.D. . . . p. 88

Tripp, John T., Ph.D. . . . p. 255, 256, *Introduction*

Trousdale, M.D. . . . p. 63, 64, 66, 69

Tullner, William, Ph.D. . . . p. 141

Uhlendorf, B. William, Ph.D. . . . p. 177, 178, 258

Wagner, John C., Sc.D. . . . p. 255, *Introduction*

West, Joseph W. . . . p. 71

Weiss, Emilio, Ph.D. . . . p. 226

Williams, Donna . . . p. 82, 83

Wolff, Sheldon, M., M.D. . . . p. 16, 17

Woodward, Mildred L. . . . p. 82

Young, Andrew M. . . . p. 82, 83

Young, Bobby G., Ph.D. . . . p. 267, 269, 271, 273, 278, 279

Zinn, Raymond D., D.V.M. . . . p. 82, 85

INDEX TO PUBLICATIONS
Division of Biologics Standards
July 1, 1967 through June 30, 1968

Pages

Albrecht, P.
Pathogenesis of neurotropic arbovirus infections.
In Current Topics in Microbiology and Immunology.
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Allen, A. M., Palmer, A. E., Tauraso, N. M., & Shelokov, A.
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208

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Density gradient ultracentrifugation of rubella virus.
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Aulisio, C. G., & Shelokov, A.
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Science (In press) 178

Subject Index

SUBJECT INDEX
Division of Biologics Standards
July 1, 1967 through June 30, 1968

AAV . . . p. 59, 67, 68, 70, 135, 136
SEE ALSO adventitious agents

abscess formation . . . p. 2, 39

adeno-associated viruses
SEE AAV

adenovirus, pathogenesis . . . p. 143

adenovirus vaccine . . . p. 213, 214

adenoviruses . . . p. 65, 129, 135, 144, 213, 214, 243, 280
Type 12 . . p. 68, 70, 135, 136, 251, 280
Type 4 . . p. 127
Type 5 . . p. 280

adjuvants . . . p. 1, 2, 16, 20, 30, 33, 34, 36, 38, 40, 72, 98, 99, 101, 135, 200, 239

adventitious agents . . . p. 1, 2, 5, 42, 43, 44, 59, 61, 63, 64, 67, 68, 70, 202, 203, 209, 215, 216, 220, 237

alcholized vaccines . . . p. 4, 52

allergic encephalomyelitis . . . p. 128

allergenic extracts . . . p. 3, 24, 26, 60
SEE ALSO specific

allergenic products . . . p. 9, 11, 15, 26, 28, 32

allergens . . . p. 95

ALS agent . . . p. 202

American hemorrhagic fever . . . p. 206
SEE ALSO hemorrhagic fever

American hemorrhagic fever viruses . . . p. 208
SEE ALSO hemorrhagic fever

animal models
SEE models

animal testing . . . p. 132, 134, 139, 141, 143, 148, 150

animal viruses . . . p. 70

anthrax vaccine . . . p. 5, 95

anti-*H. influenza* serum
SEE ALSO influenza viruses

antitoxins . . . p. 93, 95

antivenin . . . p. 96, 105

aphthae . . . p. 41, 42

arbovirus infection . . . p. 201, 205

arboviruses . . . p. 200, 204, 207, 209

Argentinian hemorrhagic fever . . . p. 207
SEE ALSO hemorrhagic fever

Argentinian hemorrhagic fever virus . . . p. 208

Arizona coral snake . . . p. 105

Arkansas Children's Colony . . . p. 171
SEE ALSO mumps virus vaccine, live

[illegible] . . . p. 1, 2, 35, 39, 40, 59, 72, 98, 100, 101, 135, 136

arthropod-borne viruses
SEE arboviruses

artificial insemenation, monkeys . . . p. 141

avian leukosis . . . p. 219

avian leucosis viruses . . . p. 128, 144, 156, 192, 209, 210, 217, 218, 220

bacterial toxins . . . p. 33, 39, 41, 43

bacterial vaccines . . . p. 13, 45, 49, 51, 54, 57, 60, 93
SEE ALSO specifific

bacterial & viral cultures . . . p. 95

BCG components . . . p. 16

BCG organism . . . p. 30, 135

BCG vaccine . . . p. 6, 20, 32

benzethonium chloride . . . p. 81

betapropiolactone . . . p. 273

bibliographies
SEE Scientific Communications

biometrics . . . p. 253

blood and blood products . . . p. 93

blood derivatives . . . p. 77, 82

blood parasites
SEE specific

blood, whole . . . p. 77

B. ovatus . . . p. 102

bovine serum albumin (BSA) . . . p. 20

B. pertussis . . . p. 4, 8, 13, 50

breeding . . . p. 141, 151

brochure
SEE Public Information

B. vulgatus . . . p. 102, 103

California encephalitis . . . p. 204, 205

canine cell cultures . . . p. 61

canine cholera . . . p. 48

canine hepatitis virus . . . p. 59, 62, 67, 68, 70

carcinogenic activity . . . p. 8, 99, 274

cashew nut oil extract . . . p. 9

catechols
SEE poison ivy extract

C. botulinum antitoxin . . . p. 96, 104

C. botulinum toxin . . . p. 96, 104

C. chauvoei . . . p. 102

Cell Culture Conference . . . p. 266

cell cultures . . . p. 191, 193, 194, 195

characterization, viruses . . . p. 66

cholera . . . p. 45

Cholera Advisory Committee . . . p. 7, 45

Cholera Panel . . . p. 7

cholera toxin . . . p. 4, 7, 45, 46, 47

cholera vaccine . . . p. 3, 6, 7, 45, 46, 47

chorioallantoic membrane . . . p. 113, 114

clam liver extract . . . p. 246, 247, 252

clinical studies . . . p. 2, 3, 9, 15, 46, 47, 154, 155, 165, 166, 170, 171

coagulation . . . p. 82, 86

cocarcinogenic activity . . . 8, 274

combined live virus vaccines
SEE specific

communications
SEE Scientific Communications

control activities . . . p. 91, 102, 104, 105, 107, 109, 111, 113, 115, 117, 118, 120, 122, 124
LBP. . . p. 5
LBE. . . p. 59, 60
LBBP. . . p. 76, 77
LCA. . . p. 91 thru 97
LP. . . p. 130
LVI. . . p. 157, 158, 159, 160
LVR. . . p. 191, 192

control testing . . . p. 91, 111, 113, 117, 118

coral snake venom . . . p. 96, 105, 140

C. tetani . . . p. 1, 35, 102

dating period . . . p. 73

demyelinization . . . p. 128

detection, viruses . . . p. 66

diagnostic reagents . . . p. 82, 93

diagnostic serums . . . p. 77

dinitrofluorobenzene (DNFB) . . . p. 23

dinitrothiocyatebenzene (DNTB) . . . p. 23

diphtheria antitoxin . . . p. 107

diphtheria toxin . . . p. 5, 95

distemper virus . . . p. 62
SEE ALSO rabies

Drakeol 6VR . . . p. 100, 101

duck hepatitis virus . . . p. 215, 216

ECHO-11 virus . . . p. 165

electron microscopy . . . p. 61, 62, 64, 66, 67, 128, 197

encephalomyelitis . . . p. 49, 85, 138

encephalopathy . . . p. 4, 49, 50

Entamoeba histolyticum extract . . . p. 28, 29

epidemic typhus vaccine . . . p. 239

epozydized soya oil . . . p. 79

ethylene glycol . . . p. 81

exchange transfusions . . . p. 276

experimental rubella vaccines, live . . . p. 169

experimental virology . . . p. 251

extracts
SEE specific

field trials . . . p. 46, 47, 166
SEE ALSO clinical studies

formalized-toxoid . . . p. 4

free fatty acids . . . p. 2, 39, 59, 101, 135, 136

freeze-drying . . . p. 94

Freund's adjuvant . . . p. 20, 28, 138

7S gamma globulin . . . p. 1, 35, 52

genetic defects . . . p. 156

granulomas . . . p. 39, 135

Guaroa virus . . . p. 204, 205

hamster tumors . . . p. 129, 251

hemorrhagic disease . . . p. 59, 69, 136

hemorrhagic fever . . . p. 136, 190, 204, 206, 207, 208

hemorrhagic fever viruses . . . p. 206, 208

hemorrhagic nephroso-nephritis . . . p. 207

Herpes-like virus . . . p. 155, 173, 174, 278

Herpes simple virus . . . p. 65, 135, 200

H. influenzae
SEE influenza viruses

histamine response . . . p. 26

histopathology . . . p. 128, 132, 134, 137, 139, 143, 146

histoplasmin . . . p. 28

house dust extract . . . p. 98

human chorionic gonadotropin . . . p. 83, 84

hydrocephalus . . . p. 172

hypersensitization . . . p. 19, 20

hyposensitization . . . p. 11, 15

identification, viruses . . . p. 66

immune serum globulin . . . p. 157, 158

Imuran . . . p. 20

inactivating agents . . . p. 81

indexing
SEE Scientific Communications

IND's . . . p. 263

infection, experimental . . . p. 136

infectious diseases, experimental . . . p. 263

infectious hepatitis, pathogenesis . . . p. 128

infectious mononucleosis . . . p. 155, 174

influenza . . . p. 240

influenza virus vaccine . . . p. 98, 189, 192, 196, 211, 212

influenza viruses . . . p. 6, 211, 238

inspection . . . p. 93

interferon . . . p. 278, 280

intracellular infection . . . p. 228

intracellular parasites . . . p. 226, 227, 228

investigational new drugs
SEE IND's

jet gun injection . . . p. 111, 155

Junin virus . . . p. 207

Kuru agent . . . p. 202

laboratory models
SEE models

latent infections . . . p. 191

L-forms . . . p. 2, 41, 42

literature searching
SEE Scientific Communications

live virus vaccine, combined
SEE specific

Maedi agent . . . p. 202

mannide monoleate
SEE Arlacel A

Marmoset diseases . . . p. 150, 151

Marmosets . . . p. 128, 150

measles immune globulin . . . p. 157

measles potency . . . p. 160

measles-smallpox vaccine . . . p. 95, 155, 159, 184

measles vaccine . . . p. 6, 130, 158, 181, 275

measles vaccine, live . . . p. 128

measles virus . . . p. 184, 200

mercurial preservative . . . p. 71

microfilaria . . . p. 151

mineral oil . . . p. 98, 99, 100, 101, 135
SEE ALSO adjuvants

models . . . p. 23, 45, 127, 200, 206, 213, 214, 222

mollusk extracts . . . p. 191

monkey cell cultures . . . p. 65

M. tuberculosis . . . p. 118

multiple antigen preparations . . . p. 93

mumps infection, natural . . . p. 170

mumps vaccine . . . p. 6, 154, 172, 192

mumps vaccine, live . . . p. 128, 144, 170

mumps virus . . . p. 127, 154, 160, 171

mumps virus, pathogenesis . . . p. 143

mycoplasma . . . p. 1, 2, 5, 42, 43, 44, 150, 161, 267, 269, 279

myelinization . . . p. 128, 137

myxoviruses . . . p. 67

national coverage
SEE Public Information

neonatal tetanus . . . p. 33

neurotropic viruses, pathogenesis . . . p. 146

neurovirulence . . . p. 128, 133, 144

neurovirulence testing . . . p. 127, 128, 130, 132, 158

New Guinea studies . . . p. 1, 38

NIH Animal Farm Center . . . p. 82, 84

nucleic acids . . . p. 226, 251
SEE ALSO viral nucleic acid

NZB mouse agent . . . p. 202

oil adjuvants
SEE adjuvants

oil emulsified antigens . . . p. 39
SEE adjuvants

oils
SEE adjuvants & specific

oleic acids . . . p. 20

olive oil . . . p. 1, 22, 35
SEE ALSO adjuvants

oncogenic activity . . . p. 131, 167, 277

oncogenic viruses . . . p. 69, 129, 135, 182, 252, 280

opacity standard . . . p. 95, 96, 109, 110

outbreak
SEE quarantine monkeys

parabens . . . p. 81

para-influenza . . . p. 65

paramyxoviruses . . . p. 67

pathogenesis . . . p. 30, 41, 129, 134

pathology . . . p. 134, 137, 139, 141, 143, 146, 148, 150

peanut oil . . . p. 1, 35, 98
SEE ALSO adjuvants

pertussis immune globulin (human) . . . p. 6

pertussis vaccine . . . p. 4, 5, 6, 7, 13, 49, 50, 138, 140

phenolized vaccines . . . p. 4, 52

phenol red . . . p. 81

phytohemagglutinin (PHA) . . . p. 20, 43

plasma . . . p. 77

plastic containers . . . p. 73, 79, 80

poison ivy extract . . . p. 3, 9, 11, 22

poison oak extracts . . . p. 9, 15

poison sumac extract . . . p. 9

polio immune globulin . . . p. 157

poliovirus . . . p. 127

poliovirus vaccine . . . p. 127, 132, 182, 183

poliovirus vaccine, live . . . p. 128, 130, 132, 158

pollen extract
SEE specific

polyoma virus . . . p. 135, 251

polyvalent vaccines . . . p. 60
SEE ALSO specific

post-vaccinal encephalitis
SEE encephalomyelitis

poxviruses . . . p. 223

preservatives . . . p. 81

Press . . . p. 283
SEE ALSO Public Information

product releases . . . p. 93

protocol reviews . . . p. 6, 91, 92

Public Information . . . p. 283

pyridine . . . p. 81

pyrogens test control . . . p. 91, 92

Q-fever rickettsiae . . . p. 239

Q-fever vaccine . . . p. 242

quarantined monkeys . . . p. 136

rabbit model
SEE models

rabbit scarification technique . . . p. 113, 114, 222

rabies virus . . . p. 200

radio . . . p. 283
SEE ALSO Public Information

ragweed extracts . . . p. 24, 25

reagent red blood cells. . . p. 77

recurrent aphthae
SEE aphthae

red blood cells, packed . . . p. 77

reference standards . . . p. 91, 93, 102, 104, 105, 107, 109, 115, 120
SEE ALSO specific 122, 124

reference toxoid . . . p. 37

reovirus . . . p. 180

respiratory viruses . . . p. 211, 214, 238, 243

rickettsial genetics . . . p. 226, 227

rickettsial seed pools . . . p. 161

rickettsial vaccines . . . p. 60,
SEE ALSO specific 93, 190, 221

RIF-free vaccine
SEE RIF-free virus

RIF-free virus . . . p. 97, 124, 192, 210, 217, 218, 219

Rous sarcoma virus . . . p. 128, 251

R. prowazeki . . . p. 136

rubella vaccine . . . p. 153, 154, 163, 166, 168, 169

rubella vaccine, safety tests . . . p. 160

rubella virus . . . p. 59, 67, 70, 127, 144, 145, 156, 163, 164, 165, 166, 167, 168

rubella virus, pathogenesis . . . p. 143

Saguinas mystax
SEE Marmosets

Schick test . . . p. 95

Schick text toxin . . . p. 5

Scientific Communications . . . p. 285, 286

scrapie agent . . . p. 202, 233

serums . . . p. 95

Simian cell culture . . . p. 64, 165

Simian hemorrhagic fever
SEE hemorrhagic fever

Simian hemorrhagic fever viruses
SEE hemorrhagic fever viruses

Simian virus 40
SEE SV40

smallpox . . . p. 155, 176

smallpox vaccine . . . p. 95, 97, 111, 113, 114, 175, 176, 190, 197, 222, 234

smallpox viruses . . . p. 158, 175, 184, 197, 222

spongiform encephalopathy agent . . . p. 202

spotted fever . . . p. 60, 72

spotted fever vaccines . . . p. 72

S. sanguis
SEE streptococci

stability, blood products . . . p. 73, 82

stabilizers . . . p. 81

sterility test, procedures . . . p. 111

storage conditions . . . p. 73, 79

streptococci . . . p. 41

S. typhosa . . . p. 4, 52, 54

SV5 . . . p. 65

SV40 . . . p. 65, 182, 183, 251

Tacaribe virus . . . p. 207

Tahyna viruses . . . p. 205

teratogenesis . . . p. 128

test systems
SEE models

tetanus antitoxin . . . p. 1, 60, 97, 115, 116

tetanus toxin . . . p. 139

tetanus toxoids . . . p. 1, 33, 37, 38, 50

therapeutic serums . . . p. 93

thimerosal . . . p. 81

tissue cultures . . . p. 161, 177, 211, 230, 232, 234, 236, 266, 267

tissue transplants
SEE transplants

toxicity . . . p. 23, 39, 40, 49, 54, 57, 139, 140

toxins . . . p. 3, 93, 95, 107

toxoids . . . p. 93, 107

transplants . . . p. 148

trypanosomes . . . p. 151

Tuberculin Conference . . . p. 7

tuberculins . . . p. 2, 5, 16, 17, 19, 20, 95, 117, 118

tuberculosis vaccine . . . p. 1, 32

tumorigenic viruses
SEE oncogenic viruses

TV . . . p. 283
SEE ALSO Public Information

Tween 80 . . . p. 170

typhoid vaccine . . . p. 4, 51, 52, 95

typhus fever . . . p. 60, 72, 136

typhus vaccine . . . p. 72, 120

ultracentrifugal studies . . . p. 83

urushiol . . . p. 3, 9, 11, 22, 23

vaccines
SEE specific

vaccinia
SEE smallpox

vaccinia virus
SEE smallpox virus

V. cholerae . . . p. 5, 45, 47, 48, 55, 56

viral diseases
SEE specific

viral genetics . . . p. 173, 182, 185, 186, 187

viral hepatitis . . . p. 156, 179, 180

viral immunology . . . p. 153, 157, 160, 161, 163, 170, 173, 175, 177, 179, 181, 182, 184, 185, 186, 187

viral nucleic acid . . . p. 185, 251

viral vaccines . . . p. 60, 64, 70, 93, 127, 143, 144, 215
SEE ALSO specific

virus biology . . . p. 246

viruses
SEE specific

Visna agent . . . p. 202

wasp agent . . . p. 122

water-in-oil
SEE adjuvants

WI-38 cells . . . p. 235, 267, 269, 271

yellow fever . . . p. 207

yellow fever vaccine . . . p. 97, 128, 144, 189, 192, 204, 209, 210, 218

yellow fever virus . . . p. 184

yellow fever virus seed . . . p. 97, 124, 192, 218

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